IDENTIFICATION OF DIFFERENTIATION MARKERS IN NORMAL AND VIRALLY TRANSFORMED AVIAN HEMATOPOIETIC CELLS

By

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DEDICATION

This dissertation is dedicated to my parents. Without their understanding, support and encouragement, it would have never been possible for me to accomplish any goals in my life.

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Words cannot express my appreciation to my mentor, Dr. Carlo Moscovici. Four years ago, I went to his office to discuss my rotation project. Suddenly I was touched by a poster on the wall, which depicted a newly hatched baby chick, excited and curious about its new life, asking " What do I do ? " It was nearly the mirror image of myself. The very same question had struck me from time to time since I arrived in the United States. Fortunately, he has guided me, spiritually and intellectually, during the past four years not only to become an independent researcher but a more mature person as well. Nevertheless, we still have some disagreement about my own differentiation pathway, e.g., he never gives up the idea that jazz should be a "growth factor" for me.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACS	Anemic chicken serum
AEV	Avian erythroleukemia virus
AHS	gamma globulin-free horse serum
ALV	Avian leukemia virus
AMV	Avian myeloblastosis virus
APAAP	Alkaline phosphatase and monoclonal anti-alkaline phosphatase
BFU-E	Burst-forming unit-erythroid
BPA	Burst-promoting activity
CEF	Chicken embryo fibroblast
CFU-E	Colony-forming unit-erythroid
CFU-M	Colony-forming unit-marrow
cpm	Counts per minute
CSF	Colony-stimulating factor
DLV	Defective leukemia virus
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein-isothiocyanate

GAM	Goat anti-mouse polyvalent IgA, G & M
HBSS	Hank's balanced salt solution
LLV	Lymphoid leukosis virus
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAV	Myeloblastosis-associated virus
MG-CFC	Macrophage-granulocyte colony-forming cell
m.o.i.	Multiplicity of infection
PBS	Phosphate-buffered solution
PMA	Phorbol 12-myristate-13-acetate
PMSF	Phenylmethylsulfonylfluoride
PNPP	*P*-nitrophenyl phosphate
PPD	*P*-phenylene diamine
RaMIG	Rabbit anti-mouse IgG
RAV	Rous-associated virus
RIA	Radioimmunoassay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TSA	Tris/saline/azide

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May 1990

Chairman: Dr. Carlo Moscovici
Major Department: Pathology and Laborotary Medicine

Avian hematopoiesis has been an excellent model for resolving numerous enigmas about growth and development. The interaction of avian retroviruses with the avian system has created a relatively new discipline of onco-development which allows us to analyze abnormal tissue growth and hematological disorders in a more sophisticated fashion.

The specific aim of this project is to identify lineage-specific differentiation markers in normal avian hematopoietic cells and transformation-associated antigens in retrovirus-transformed cells by utilizing monoclonal antibody techniques. Four groups of MAbs were selected among nearly 5,000 supernatants from 10 fusions. Characterization of the cell-type specificities was achieved

by radioimmunoassay, immunofluorescence staining, flow cytometry and immunoenzymatic staining as well as FACStar sorting or immunomagnetic bead separation followed by colony-forming assays and transforming assays. Analysis of MAbs revealed that 1) MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 are specific for transformation-associated antigens present preferentially on BM2 cell lines rather than on normal monocytic cells. The expression of these antigens was diminished after BM2 cells were induced to differentiate. 2) MAb 1F7-1A3 recognizes BFU-E and CFU-E, AEV-transformed yolk sac cells and MSB1 cells. 3) MAb 3F6-1E7 reacts with the embryonic stem cell and precursor cell populations. The expression of the marker recognized by MAb 3F6-1E7 was also observed on some tumor cells, *e.g.*, AEV-transformed yolk sac cells, BM2 cells and MSB1 cells. 4) MAb 2E10-1E10 defines a marker present on proliferating hematopoietic cells instead of terminally differentiated cells, however, it starts appearing only after the 4th day of embryogenesis.

Trypsinization, neuraminidase digestion and deglycosylation treatment reduced the binding specificities of MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10. This suggests that the markers recognized by these MAbs are glycoproteins and that sialic acids with or without carbohydrates are contributing to the conformation of the antigenic determinants. Conversely the antigenic

determinants for MAbs 1F7-1A3 and 3F6-1E7 must be strictly proteins, since only trypsinization was able to inhibit their binding specificities.

This study will permit investigations focusing on the expression of these markers to bring us a step closer toward the understanding of the mechanisms involved in regulating proliferation and differentiation of normal cells versus tumor cells.

CHAPTER 1

INTRODUCTION AND BACKGROUND

Introduction

Proliferation and differentiation of normal cells is controlled by the interactions with other cell types, with extracellular matrix and with regulatory molecules such as growth factors and differentiation factors. Although the regulatory mechanisms are extremely complicated, they have been programmed in such a way as to maintain proliferation and differentiation of the cells in a harmonic state. In other words, the loss of cells from the stem cell compartment by differentiation into committed progenitor cells must be balanced by replenishment via self-renewal of the stem cells. If too many stem cells undergo differentiation, the stem cell reserve will rapidly become exhausted; if too many stem cells undergo self-renewal rather than differentiation, the production of mature cells will drastically fall.

Cancer is believed to be a molecular disease resulting from the deregulation of proliferation and differentiation, *i.e.*, the harmony has been short-circuited by the

constitutively triggered self-renewal machinery with or without the blockage of the differentiation pathway.

It has been noticed that AEV-transformed avian embryonic yolk sac cells can eventually undergo spontaneous differentiation into mature erythrocytes (Jurdic *et al.*, 1985) and that spontaneous regression and differentiation of human neuroblastomas are observed occasionally (Evans *et al.*, 1976). In addition, a variety of tumor cells have also been shown to revert to a normal state under different conditions despite the continued expression of activated oncogenes. For example, the tumorigenicity of hybrids formed between normal and tumor cells has been completely suppressed (Stanbridge *et al.*, 1982); embryonal carcinoma (Pierce *et al.*, 1979), neuroblastoma (Podesta *et al.*, 1984), B16 melanoma (Pierce *et al.*, 1984) and murine leukemia (Gootwine *et al.*, 1982) have been converted into benign cell lineages by their appropriate embryonic environments; naturally occurring substances such as colony-forming factors (Sachs, 1986), glia maturation factor (Lim *et al.*, 1986) and transforming growth factors (Sporn *et al.*, 1986) have been shown to be able to induce differentiation of tumor cells; while a number of chemical agents such as hexamethylene bisacetamide, retinoid acid, 5-azacytidine and DMSO etc. can also induce terminal differentiation and/or reverse the neoplastic phenotype of malignant cells (Bloch, 1984; Fresney, 1985). Moreover, in some cases, terminally

differentiated cells such as macrophages can still serve as the target cells for transformation by a group of retroviruses, namely AMV, MC29 and MH2 (Pessano *et al.*, 1979). All the information mentioned above suggests that self-renewal and differentiation of cells are regulated by separate mechanisms. The roles of various protooncogenes and antioncogenes in these processes are yet to be elucidated.

Normal Avian Hematopoiesis

Introduction

The avian hematopoietic system has provided a unique and interesting model to study mechanisms of the regulation of cell proliferation and differentiation in normal versus tumor cells. It has several distinct features compared to that of mammals, including the presence of the bursa of Fabricius which is involved in the differentiation of B lymphocytes, the expression of class IV MHC antigens, coded by the B-G region, on the surface of the mature erythrocytes (Miller *et al.*, 1982). In addition, the erythrocytes are nucleated and oval-shaped, and the nucleated thrombocytes, instead of the platelets, are responsible for the hemostasis in the avian system. There are several advantages in using the avian models. For instance, tolerance to foreign antigens can be developed during early ontogeny (Hasek and Hraba, 1955), and hematopoiesis can be studied both *in vivo*

and *in vitro* by using retroviruses as indicators for specific precursors present within each lineage.

The most studied avian hematopoietic system is in the chicken. The outbred SPAFAS line has been used in our laboratory to carry out all the experiments for my dissertation project. My work has focused mainly on the erythroid and monocytic lineage and their interaction with the avian retroviruses.

The Blood-Forming Organs

The first blood cells appear after 18 hours of incubation in the blood islands disseminated in the blastoderm. During embryogenesis, the yolk sac represents the major hematopoietic organ until day 15. Erythropoiesis in the spleen starts around day 9 and continues through day 16 to 18 with a peak at day 15. The bone marrow begins its function on day 12 and becomes the main site of hematopoiesis throughout adult life (Dieterlen-Liévre, 1988).

The yolk sac

The yolk sac becomes established during the third day of embryogenesis and it originates in the extraembryonic region consisting of a peripheral "*area vitellina*" which is made up of ectoderm and endoderm only, and a central "*area*

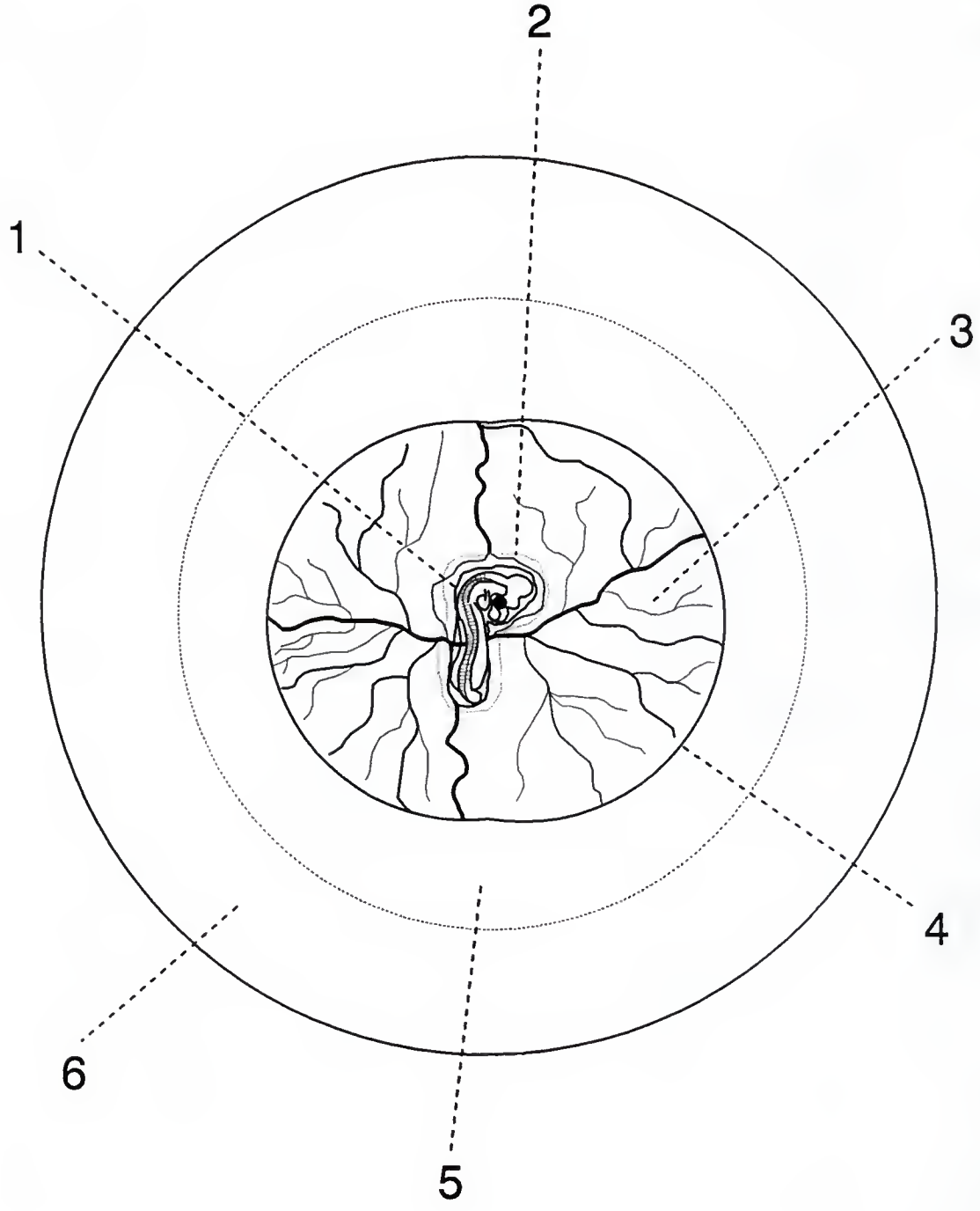
vasculosa" consisting of all three germ layers (Figure 1-1). The area vasculosa contains the blood islands and its boundary is delineated by a circular blood vessel, the sinus marginalis. As the area vitellina grows over the yolk, the area vasculosa also increases in size and invades the area vitellina. Eventually the latter disappears completely, and the entire yolk sac is vascularized.

The bone marrow

In the adult chicken, the bone marrow remains the major source for erythropoiesis, granulopoiesis and lymphopoiesis. The spleen does not appear to play a role in hematopoiesis in the adult. In the avian bone marrow, erythropoiesis occurs in the lumen of the medullary sinuses, while granulopoiesis and lymphopoiesis are compartmentalized within the extravascular spaces (Campbell, 1967). In addition, masses of lymphatic tissue with germinal centers are also present (Campbell, 1967; Payne and Powell, 1984). However, in the mammalian bone marrow, erythropoiesis is confined to the extravascular spaces and there is no lymphatic tissue at all.

The development of the bone marrow during ontogeny has been studied in the chick embryo by Sorrell and Weiss (1980) using light, scanning and transmission electron microscopy. The bone marrow cells can be obtained from the chick embryo as early as 12 days of incubation. Marrow at this stage is

Figure 1-1. Schematic illustration of the chick embryo at the 3rd day of incubation. The yolk sac is composed of the area vitellina and the area vasculosa. As the area vitellina grows over the yolk, the area vasculosa also increases in size and invades the area vitellina. Eventually the latter disppears completely and the entire yolk sac is vascularized. 1, embryo; 2, area pellucida; 3, area vasculosa; 4, sinus terminus; 5, area vitellina interna; 6, area vitellina externa.


2
1
3
4
6
5

richer in stem cells and non-committed progenitor cells than the older bone marrow, but it already harbors cells which are committed to specific hematopoietic lineages. The latter cells are less numerous than in the adult bone marrow and consist essentially of hemocytoblasts.

Other blood-forming organs

The spleen, the bursa, the liver and the thymus function as additional hematopoietic organs. In the adult, lymphopoiesis occurs mainly in the thymus and in the spleen, whereas the bursa, from which the precursors of B lymphocytes originate, is a transient granulopoietic organ.

Recent investigations (Cormier and Dieterlen-Liévre, 1988) report that some intraembryonic sites may be another source of hematopoietic stem cells in the developing embryo. At 3-4 days of incubation, the wall of the dorsal aorta surrounding the intraembryonic mesenchyme is found to be the site from which hematopoietic progenitor cells emerge, *i.e.*, M-CFC, G-CFC, GM-CFC and BFU-E.

The Differentiation Pathway of Hematopoietic Cells

The cells of the hematopoietic system arise by proliferation and differentiation of the progenitor cells. This process begins with multipotential stem cells which can self-renew as well as undergo progressive differentiation to progenitor cells committed to the particular lineages,

ultimately yielding mature blood cells (Metcalf and Moore, 1971).

Analysis of the stem cells and progenitor cells in the different hematopoietic tissues has been useful in clarifying the differentiation pathway as well as in exploring the regulatory mechanisms of hematopoiesis.

Hematopoietic stem cells

The stem cell population is the fundamental base from which all the major hematopoietic cell lines are derived. This population is thus considered to be pluripotent in its differentiation potential, giving rise to erythroid, granulocytic, monocytic, megakaryocytic and lymphoid lineages (Figure 1-2). However, the stem cells account only for 0.01% of the total bone marrow cells in a normal mouse, and for 0.003-0.004% in a normal chicken (Table 1-1). In addition, since they are morphologically indistinguishable, their existence can only be inferred by the progeny they produce.

Efforts to identify chicken hematopoietic stem cells have followed the protocol of the transplantation experiments by Till and McCulloch (1961) whereby they have identified the mouse hematopoietic stem cells. Samarut, *et al.* (1976) transplanted normal chicken bone marrow into irradiated chickens. Six days later, erythrocytic colonies were observed only on the surface of the tibial marrow.

Figure 1-2. Diagram of hematopoiesis. The entire pool of mature hematopoietic cells is derived from a single pluripotent stem cell. The more differentiated they become, the less self-renewal potential they possess. In the avian system, thromboblast and thrombocytes stand for megakaryocyte and platelets respectively. Abbreviations used: BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; Eo-CFC, eosinophil-colony forming cell; GM-CFC, granulocyte & monocyte-colony forming cell; MEG-CFC, megakaryocyte-colony forming cell.

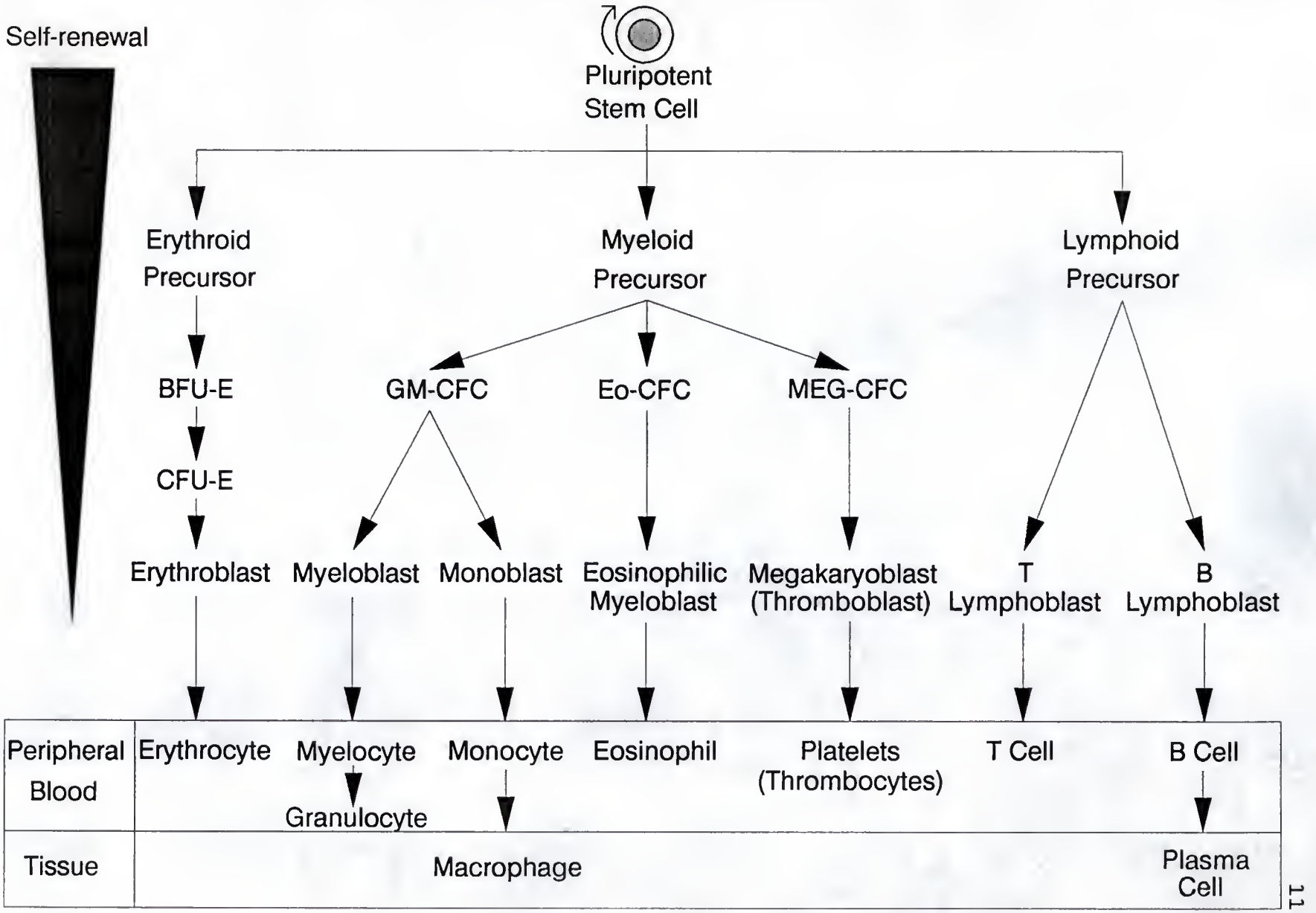

Self-renewal
Pluripotent Stem Cell
Erythroid Precursor
Myeloid Precursor
Lymphoid Precursor
BFU-E
CFU-E
GM-CFC
Eo-CFC
MEG-CFC
Erythroblast
Myeloblast
Monoblast
Eosinophilic Myeloblast
Megakaryoblast (Thromboblast)
T Lymphoblast
B Lymphoblast
Peripheral Blood
Erythrocyte
Myelocyte
Granulocyte
Monocyte
Eosinophil
Platelets (Thrombocytes)
T Cell
B Cell
Tissue
Macrophage
Plasma Cell

Table 1-1. Chicken hematopoietic precursor cells

Hematopoietic precursor cells	Frequency*		Progeny
	Bone marrow	Yolk sac	
BFU-E	110-160	300-600	1,000-2,000
CFU-E	500-2,000	1,500-1,800	8-150
GM-CFC (early progenitor)	250-400	100-200	50-1,000
GM-CFC (late progenitor)	1,000-1,300	100-200	3-50

Source: Modified from Moscovici and Gazzolo (1982).
*Expressed as per 10^5 cells.

Each colony was originated from one single cell, *i.e.*, colony-forming unit in the marrow (CFU-M). However, neither macrophage-granulocytic colonies nor mixed types of colonies were observed in the marrow of the irradiated chickens. It is still unclear whether the medullar environment does not favor the development of colonies other than the ones of the erythroid lineage or if the CFU-M represents only the stem cells at the earliest step of commitment in the erythroid lineages.

Committed progenitor cells

Committed progenitor cells are directly derived from the stem cells and are each committed to a specific differentiation pathway or lineage. Commitment is an irreversible step whereby these cells have lost the potential to generate hematopoietic cells of other lineages. Most of the proliferative activity in the bone marrow seems to occur in the progenitor cells committed to the production of single or restricted ranges of hematopoietic cell types. Only a small proportion of the pluripotential stem cells is cycling at a given time. The progenitor cells of both erythroid and myeloid lineages will be discussed in detail.

The erythroid lineage

The avian erythroid compartment consists of three distinct lineages, namely the primitive, the intermediate

and the definitive lineage, respectively (Table 1-2). The cells from the primitive lineage are produced by the early blood islands and mature "in cohort" (Ingram, 1972). These cells are released at a very immature state, but they continue to divide and mature synchronously within the blood vessels between 2 and 5 days of incubation. These cells are called megalocytes because of their large size. They are spherical with round nuclei and synthesize hemoglobins E (embryonic) and P (primitive) which are specific for the primitive lineage. At 5 days, cells of the intermediate erythroid lineage begin entering the blood and eventually supersede the primitive cells. At 7 days, the primitive cells account for only 5% of the red blood cells, and after 12 days of incubation they are rarely encountered. The cells of the intermediate lineage are observed from 5 to 6 days until 18 to 20 days. They synthesize specific hemoglobin H (hatching). It is not until 18 to 21 days of incubation that mature erythrocytes of the definitive lineage start to appear in the blood circulation. They are oval-shaped with oval nuclei and have hemoglobins A (adult) and D (definitive) (Bruns and Ingram, 1973).

The progenitor cells of the definitive lineage are morphologically unrecognizable. However, by the use of *in vitro* colony forming assays in methylcellulose, two classes of erythroid progenitor cells have been identified, the colony-forming unit-erythroid (CFU-E) and the burst-

Table 1-2. The three different lineages of the avian erythroid compartment

	Primitive	Intermediate	Definitive
Appearance	Day 2-7	Day 5-20	Day 18-Hatched
Progenitor Cell	Megaloblast?	BFU-E, CFU-E	BFU-E, CFU-E
Mature Cell	Megalocyte	Erythrocyte	Erythrocyte
Hemoglobin*	E: $\alpha^A + \epsilon$ P: $\pi + \rho$	H: $\alpha^A + \beta^H$	A: $\alpha^A + \beta^A$ D: $\alpha^D + \beta^A$

*Source: Modified from Bruns and Ingram (1973).
α-like globin: α^A, π, α^D; β-like globin: ϵ, ρ, β^H, β^A.
Abbreviation: E, embryonic; P, primitive; H, hatching; A, adult; D, definitive.

forming uint-erythroid (BFU-E). The BFU-E give rise, after six days in culture, to large aggregates made of several benzidine-positive clusters containing about 1,000 erythrocytes (Samarut and Bouabdelli, 1980). These BFU-E are highly sensitive to burst-promoting activity (BPA), and are also dependent on high concentrations of erythropoietin.

The CFU-E proliferate to form one compact colony of 8 to 150 benzidine-positive erythrocytes after three days of incubation (Samarut *et al.*, 1979). The requirement for erythropoietin in the development of CFU-E is lower than that for BFU-E. The BFU-E and CFU-E can be detected in the yolk sac as well as in the embryonic and adult bone marrow. The BFU-E are also found in the blastoderm at the primitive streak stage (18 hours of incubation), whereas the CFU-E are not yet detectable (Samarut and Bouabdelli, 1980). The BFU-E and CFU-E can be also distinguished by the expression of two different cell surface antigens which are recognized by polyclonal antisera (Gazzolo *et al.*, 1980; Samarut *et al.*, 1979). An antigen specific to immature red blood cells is present on the CFU-E but not detectable on the BFU-E. Conversely, a chicken brain-related antigen is expressed on the BFU-E and less expressed on the CFU-E.

In the murine system, subpopulations of the BFU-E at different degrees of maturation have been observed (Gregory and Eaves, 1978). This is not the case, however, for the BFU-E in the chickens.

The myeloid lineage

Hematopoietic cells of granulocytic and monocytic lineages are referred to as myeloid cells. In response to infection, the progenitor cells in the bone marrow, *i.e.*, granulocytic-macrophage colony-forming cells (GM-CFC), would rapidly produce a large amount of mature granulocytes and monocytes under the control of a variety of colony-stimulating factors (CSFs). In the murine and human system, four kinds of CSFs involved in myelopoiesis have been characterized as IL3, GM-CSF, G-CSF and M-CSF. Conversely, the specific CSFs in the chicken have yet to be identified. Nevertheless, sources of CSFs can be furnished by using an underlayer of macrophages (Graf *et al.*, 1981), or by adding to the semi-solid medium either egg albumin (Szenberg, 1977), or serum from endotoxin-injected chickens (Dodge and Hansell, 1978) or a conditioned medium from chicken fibroblast cultures (Dodge and Moscovici, 1973; Dodge *et al.*, 1975; Gazzolo *et al.*, 1979). AMV-transformed cells were shown also to be capable of producing CSFs (Silva *et al.*, 1974). Recently a myelomonocytic growth factor was isolated from medium conditioned by a transformed macrophage cell line (Leutz *et al.*, 1984). This factor (MGF) promotes the growth of macrophage colonies together with a minor proportion of granulocytes.

The existence of chicken GM-CFC can be detected when bone marrow cells are cultured in soft agar or

methylcellulose media. The wide range of the colony size obtained suggests that these progenitor cells display different degrees of maturity. The less mature CFC give rise to clusters containing from 50 to more than 2,000 cells (Dodge and Moscovici, 1973; Dodge *et al.*, 1975), while the more mature CFC produce colonies from 3 to 50 cells (Gazzolo *et al.*, 1980). Colonies which are composed mostly of macrophages readily develop in a semi-solid medium containing chicken serum and fibroblast-conditioned-medium (Dodge and Hansell, 1978). Granulocytic colonies will develop if the chicken serum is depleted from the medium and the fibroblast-conditioned-medium is replaced by spleen-conditioned-medium (Dodge and Sharma, 1985). This finding suggests the existence of a factor similar to M-CSF in the mouse and human. The monocytic colonies are composed of scattered cells, whereas the granulocytic colonies are dense. The cells from both colonies contain granules.

The GM-CFC have been observed in various chicken hematopoietic tissues of embryonic and adult stages (Dodge and Moscovici, 1973; Dodge *et al.*, 1975; Szenberg, 1977). Moreover, the CFC can be enumerated in the blastoderms incubated for 24 hours (Moscovici *et al.*, unpublished results). A higher percentage of CFC was also found in the embryonic spleen and bone marrow. The frequency dropped rapidly once the chickens hatched. Interestingly, a peak of CFC occurred in the bursa at 14 and 15 days of incubation,

indicating that the stem cells colonizing the bursa differentiate first into myeloid elements and subsequently into lymphoid ones (Szenberg, 1977).

Interaction of the Avian Leukemia Viruses with Hematopoietic Cells

Introduction

There have been many comprehensive reviews to date on the molecular biology and the pathogenesis of the avian leukemia viruses (ALVs) (Moscovici and Gazzolo, 1982; Graf and Stéhelin, 1982; Bishop, 1983; Enrietto and Wyke, 1983; Bister and Jansen, 1986). The ALVs belong to a taxonomic subfamily termed oncovirinae (specifically, avian leukosis-sarcoma group of type C RNA tumor viruses) within the family of retroviridae (retroviruses) (Fenner, 1976). They contribute to a variety of avian hematopoietic as well as non-hematopoietic disorders. The ALVs can be divided into two groups according to the pathological features: the defective leukemia viruses (also known as acute leukemia viruses) and the avian leukosis viruses (Hanafusa, 1977).

The defective leukemia viruses (DLVs)

These viruses induce various types of acute leukemia within a few weeks after inoculation. They also cause sarcomas and carcinomas in some cases. All the strains known can transform the cells of specific hematopoietic lineages *in vitro*. In addition, most of them transform

fibroblasts in culture as well, with the exception of AMV and E26. Another distinct aspect of the DLVs is that they are all replication-defective due to total or partial deletions of the essential virion genes: *gag, pol* and *env*. Consequently, they can produce infectious progeny only in the presence of the avian leukosis helper viruses. The deleted sequences are replaced by the viral oncogenes (v-*onc*), *i*.e., v-*myc*, v-*mil*, v-*erbA*, v-*erbB*, v-*myb*, and v-*ets*, which originated from transduction of mutated or truncated forms of protooncogenes. Their gene products are responsible for the transformation of the hematopoietic cells. Based on the predominant response of the hematopoietic system of the infected host and the major types of oncogenes which they carry, three subgroups of DLVs and their respective representatives can be distinguished: i) the MC29 subgroup: myelocytomatosis (v-*myc*), ii) the AEV subgroup: erythroblastosis (v-*erb*), iii) and the AMV subgroup: myeloblastosis (v-*myb*) (Table 1-3). However, a more detailed description of the interaction of the AEV and the AMV with the hematopoietic system will be given since more data have been obtained in the last decade.

The Lymphoid leukosis viruses (LLVs)

In contrast to the DLVs, the LLVs do not contain any v-*onc* and they are fully competent for replication. Because

Table 1-3. Avian defective leukemia viruses

Subgroups and virus strains	Viral oncogenes	Neoplasms induced *in vivo*	Cell types transformed *in vitro*
MC29 subgroup			
Strain MC29	v-*myc*	Myelocytomatosis,	Myeloid,
Strain CMII	v-*myc*	endothelioma,	macrophage,
Strain OK10	v-*myc*	carcinoma	epithelioid,
Strain MH2	v-*myc*, v-*mil*		fibroblastic
AEV subgroup			
Strain AEV-R	v-*erbA*, v-*erbB*	Erythroblastosis,	Erythroid,
Strain AEV-H	v-*erbB*	fibrosarcoma	fibroblastic
AMV subgroup			
Strain AMV	v-*myb*	Monoblastosis	Monocytic
Strain E26	v-*myb*, v-*ets*	Erythroblastosis, monoblastosis	Erythroid, monocytic

Source: Modified from Bister and Jansen (1985).

of the absence of v-*onc*, the LLVs don't transform cells *in vitro* and most strains induce predominantly lymphoid leukosis *in vivo* only after a long latent period of several months or longer by the activation of protooncogenes. In the cases of chicken B-cell lymphomas induced by the LLVs, the viral LTR regions containing an enhancer and a promoter were found to be integrated very close to the c-*myc* gene (Hayward *et al.*, 1981). Most integrations of LLVs result in the separation of exons II and III of the c-*myc* gene from the normal promoter and exon I (Payne *et al.*, 1982; Shih *et al.*, 1984), causing a fifty-fold higher transcription of c-*myc* RNA under the control of the LTR promoter than the 5 copies found in normal cells. In a minority of tumors, the LTR integrated in the opposite orientation to that of the c-*myc* and in one case the provirus was actually located at the 3' end of the c-*myc* (Payne *et al.*, 1982). It is thought in these rare events that the enhancer element in the viral LTR probably increases the transcription from the normal c-*myc* promoter. More recently, the LLVs have also been found integrated at the c-*erbB* locus in chicken erythroblastosis (Fung *et al.*, 1983). An elevated level of c-*erbB* transcripts was observed.

LLVs under different conditions, for example, chicken genotype, site of injection, age of host, etc., may induce a larger spectrum of diseases including osteopetrosis, anemia,

nephroblastoma and occasional fibrosarcomas and endotheliomas.

The Avian Erythroleukemia Virus (AEV)

The oncogenes of the AEV

The AEV can induce erythroblastic leukemia and sarcomas in infected birds within a short period of time (Graf and Beug, 1978; Moscovici and Gazzolo, 1982). The virus also transforms chicken fibroblasts and hematopoietic precursor cells of the erythroid lineage *in vitro* (Graf *et al.*, 1981). It carries two oncogenes, namely v-*erbA* and v-*erbB*. The v-*erbB* encodes a protein of 61 Kd which is glycosylated in infected cells to higher molecular weight forms (Privalsky *et al.*, 1983). The $gp65^{erbB}$ and $gp68^{erbB}$ are localized in the intracellular membrane, while the mature $gp74^{erbB}$ is found in the plasma membrane (Privalsky and Bishop, 1984). The latter protein represents a truncated form of the receptor for epidermal growth factor (EGF) (Downward *et al.*, 1984), where the extracellular EGF-binding domain has been deleted, but the region for tyrosine-specific protein kinase is preserved (Gilmore *et al.*, 1985). It has been postulated that the transforming potential of v-*erbB* is due to the lack of regulation by EGF resulting in the constitutive activation of the tyrosine kinase (Hayman and Beug, 1984; Privalsky and Bishop, 1984). However, the exact mechanism

of transformation by the tyrosine kinase activity is still unknown.

The v-*erbA* is a mutated truncation transduced from a cellular multigene family encoding the thyroid hormone receptor (Sap *et al.*, 1986). The v-*erbA* protein, $p75^{gag\text{-}erbA}$, is linked with the *gag* product, is unglycosylated and has no kinase activity. It appears to be a DNA-binding protein exhibiting distinct nuclear and cytoplasmic subcellular locations (Boucher *et al.*, 1988). Nevertheless, it doesn't seem to bind thyroid hormone (Sap *et al.*, 1986).

The transforming potential of v-*erbA* and v-*erbB*

It has been shown that v-*erbB* alone is necessary and sufficient to induce cell transformation (Frykberg *et al.*, 1983; Sealy *et al.*, 1983) by constructing a series of deletion mutant viruses. The data corroborate the ability of the product of v-*erbB* $gp65\text{-}68^{erbB}$ to independently induce erythroleukemia in chickens and transform fibroblasts *in vitro*. In contrast, constructs which produce the v-*erbA* $p75^{gag\text{-}erbA}$ only were incompetent for transforming activity *in vitro*. However, other observations from studies of temperature sensitive mutants (Beug and Hayman, 1984), transductants of the c-*erbB* gene alone (Fung *et al.*, 1983; Yamamoto *et al.*, 1983), suggest that v-*erbA* may play a distinct role in maintaining the proliferation and transformed phenotype of AEV-infected cells. It has been

shown that v-*erbA* possesses the capability to potentiate the erythroid transformation not only by v-*erbB* but also by other oncogenes (Frykberg *et al.*, 1983; Kahn *et al.*, 1986). These phenomena could be partially due to the suppression of the transcription of the anion transporter (band 3) gene by the v-*erbA* proteins (Zenke *et al.*, 1988). It is not until recently, however, that the transforming potential of v-*erbA* has been reevaluated. The XJ12 vector which carries v-*erbA* oncogene in association with Neo R (neomycin resistance) gene is shown to be able to transform bone marrow cells *in vitro* (Gandrillon *et al.*, 1989; Moscovici and Moscovici, unpublished results).

The target cells for AEV

The target cells for AEV infection in the bone marrow of the hatched chick are recruited within the BFU-E (Burst forming unit-erythroid) compartment (Gazzolo *et al.*, 1980). After AEV infection, the transformed cells continue to proceed within the differentiation pathway until they are blocked at the stage of CFU-E (colony forming unit-erythroid) (Samarut and Gazzolo, 1982). These cells express the erythroid markers of the CFU-E, but have gained the self-renewal potential to forego the fate of terminal differentiation. Conversely, the target cells for AEV in the embryo are within either the CFU-M or the pre-BFU-E or in both compartments (Jurdic *et al.*, 1985). The embryonic

transformed colonies are partially hemoglobinized even after subcloning, which suggest that the AEV-transformed embryonic cells can escape the blockage and undergo spontaneous differentiation in contrast to the AEV-transformed adult cells (Figure 1-3).

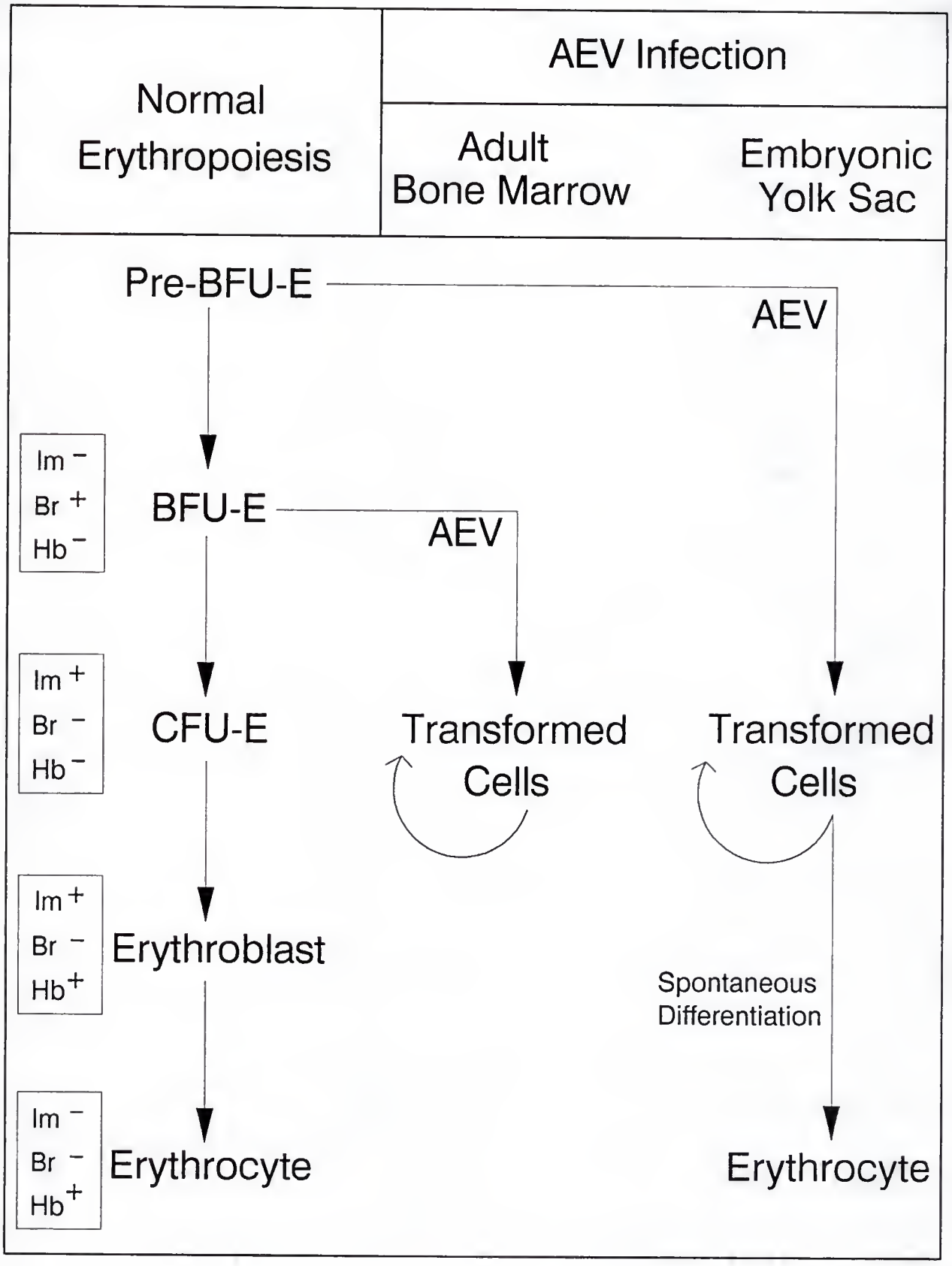
The Avian Myeloblastosis Virus (AMV)

The oncogene of the AMV

The AMV induces rapid myeloblastic leukemia in the chickens and transforms hematopoietic cells of monocytic lineages *in vitro*. It can be divided into subgroups A and B, depending on the expression of envelope glycoproteins of the helper viruses (Moscovici *et al.*, 1975). Besides the AMV transforming agent, this strain contains two nondefective helper leukosis viruses, the myeloblastosis associated virus 1 and 2 (MAV-1 & MAV-2) of subgroups A and B, respectively (Moscovici and Vogt, 1968).

The oncogene of AMV, v-*myb*, encodes a transforming protein $p45^{myb}$ which is located in the nucleus (Klempnauer *et al.*, 1984). It has been shown that $p45^{myb}$ has DNA- binding activity (Boyle *et al.*, 1985). The v-*myb* proteins are associated with their nuclear sublocation. The exact function of v-*myb* proteins remains to be clearly established. However, recent results indicate that v-*myb* along with c-*myb* may act as transcriptional activators (Weston and Bishop, 1989).

Figure 1-3. Interference of the AEV with cells of the erythroid lineage. The AEV only transforms the cells at the BFU-E stage from the adult bone marrow. Transformed cells are frozen at the CFU-E stage and capable of self-renewal. Only cells at the pre-BFU-E stage are the target cells for AEV transformation in the embryonic yolk sac. Although the transformed cells display the phenotypes of CFU-E, they will eventually escape the blockage and spontaneously differentiate into erythrocytes. The brain antigens (Br) are expressed on the BFU-E more than on the CFU-E, the immature antigens (Im) are found on the CFU-E and erythroblasts, meanwhile only erythroblasts and erythrocytes are capable of synthesizing the hemoglobins (Hb).


Normal Erythropoiesis
AEV Infection
Adult Bone Marrow
Embryonic Yolk Sac
Pre-BFU-E
AEV
Im −
Br +
Hb −
BFU-E
AEV
Im +
Br −
Hb −
CFU-E
Transformed Cells
Transformed Cells
Im +
Br −
Hb +
Erythroblast
Spontaneous Differentiation
Im −
Br −
Hb +
Erythrocyte
Erythrocyte

The target cells for the AMV

The characterization of AMV target cells by density, velocity sedimentation, adherence and phagocytic activity indicate that they are recruited among a wide range of cells within the monocytic lineage from the stage of the myelomonocytic progenitors, *i.e.*, CFC (colony-forming cells) (Gazzolo *et al.*, 1979) committed toward the macrophage lineage (Boettiger and Durban, 1984) to the terminally differentiated macrophages (Moscovici and Gazzolo, 1982). Regardless of the origin of the target cells, the AMV-transformed cells are morphologically identical, and possess the same functional and surface properties (Gazzolo *et al.*, 1979; Beug *et al.*, 1979; Durban and Boettiger, 1981). They are mostly nonadherent and round which diametered about 10 micrometers. Their large and eccentric nucleus is surrounded by a rim of cytoplasm containing small granules. The receptors for the Fc region of immunoglobulins are expressed on the cell surface, whereas C_3 receptors are not present. Normal avian macrophages express both receptors. Although the AMV-transformed cells can engulf latex particles mediated by nonspecific receptors, phagocytosis mediated by Fc receptors does not occur, *i.e.*, these Fc receptors are not functional. Acid phosphatase and adenosine triphosphatase are also found in the cytoplasm and on the membrane of the transformed cells. Moreover, after treatment with phorbol 12-myristate-13-acetate (PMA), a

tumor promoter, the AMV-transformed cells became adherent to the surface of the culture flask and eventually differentiated into macrophages (Pessano *et al.*, 1979). There were no obvious alterations in terms of the expression of the v-*myb* proteins in these PMA-differentiated cells. However, the v-*myb* proteins were found to be located in the cytoplasm instead of in the nucleus (Symonds *et al.*, 1984). The differentiation into macrophages was also obtained with a temperature-sensitive mutant of AMV when the transformed cells were shifted to a non-permissive temperature (Moscovici and Moscovici, 1983).

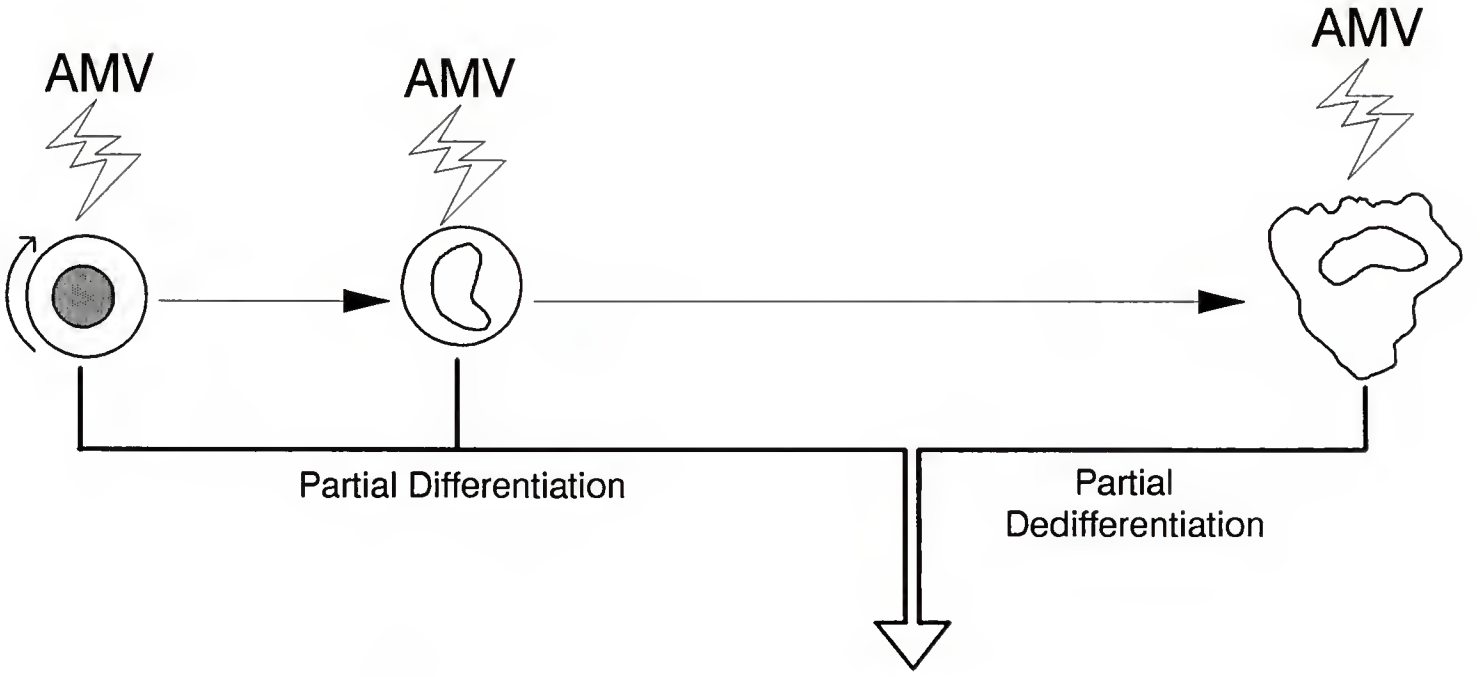
In conclusion, cells from all stages of monocytic lineages, from the committed progenitors to the mature macrophages, may serve as the target cells for AMV. Once the cells are transformed, they become frozen at a stage between monoblasts and monocytes (Figure 1-4).

Identification of Cell Surface Markers in Normal Hematopoietic Cells and Tumor Cells by MAbs

Introduction

The cellular microenvironment plays a crucial role in regulating the proliferation and differentiation of normal cells. A cell will interact with adjacent cells and with structural components of the extracellular matrix via the external surface of its plasma membrane. Although little is known about the mechanisms of cellular interactions, it is well established that cell development requires that the

Figure 1-4. Interference of the AMV with cells of the monocytic lineage. Cells from different stages of the monocytic lineage can serve as the target cells for AMV transformation. The transformed cells are arrested at the stage between monoblast and monocyte via either partial differentiation or de-differentiation. These cells are non-adherent and round-shaped. The ATPases are expressed on their cell surface, whereas C3 receptors are not. Although they have Fc receptors, no immune-phagocytosis can be mediated by these non-functional receptors.



GM-CFC	Monoblast	AMV transformed cells	Macrophages
Non-adherent	Non-adherent	Non-adherent	Adherent
	Fc Receptor −	Fc Receptor +	Fc Receptor +
	C3 Receptor −	C3 Receptor −	C3 Receptor +
	Phagocytosis −	Immune Phagocytosis −	Immune Phagocytosis +
		ATPase +	ATPase +

surface membrane should be capable of receiving and transmitting regulatory signals, *i*.e., growth factors and differentiation factors from the microenvironment. Cancer is a disease resulting from abnormalities in both cell proliferation and differentiation characterized by increased growth rate, prolonged survival, decreased adhesion, loss of contact inhibition, increased invasiveness and motility, expression of repressed antigens and escape from immune surveillance (Wallach, 1968). All these phenomena have been shown to be associated with alterations in structure and function, and in particular with an aberrant glycosylation of the cell surface membrane. As a result, cancer can be regarded as a molecular disease of cell surface glycoconjugates (Abe *et al*., 1983).

Cell surface glycoconjugates comprise a heterogeneous group of compounds, all of which contain carbohydrate *N*-glycosidically or *O*-glycosidically linked to protein (glycoproteins) or *O*-glycosidically to lipid (glycolipids). The predominant glycoconjugates are glycoproteins containing at least 80% of all cell surface-located carbohydrate. It is assumed that all membrane-bound proteins but only 10% of membrane lipids are glycosylated (Shinitzky, 1984). However, it is extremely difficult to identify specific glycoconjugates expressed exclusively on tumor cell surface. All identified tumor-associated markers to date are found to

be more or less expressed on normal cells at particular stages of the differentiation pathway (Old, 1981).

The identification and characterization of cell surface markers can provide us with invaluable information on various aspects of differentiation and oncogenesis. First, the identification of cell surface markers that are specific for particular stages of differentiation and maturation will enable us to follow the differentiation pathway in molecular terms. Mechanisms regulating the expression of cell surface markers will allow us to understand how functionally mature cells are formed and what kind of structural elements are necessary for functional differentiation. Secondly, they could be used to study whether tumor cells are arrested at a certain stage of differentiation. In addition, the studies could also enable us to determine how the control of proliferation and differentiation in tumor cells differs from that of normal cells. Thirdly, differentiation markers can be used for diagnostic and therapeutic purposes (Fukuda, 1985).

The introduction of hybridoma technology by Kohler and Milstein (1975) has made a significant contribution in the study of both normal and malignant cell surface markers. MAbs have been used as powerful tools for the detection, isolation and characterization of cell surface markers. Compared to polyclonal antibodies, MAbs exhibit three major advantages. First, they can be produced against relatively

impure antigens. Secondly, MAbs can be produced in much larger (theoretically unlimited) quantities. Thirdly, they are monospecific (*i*.e., they bind to a single epitope) and thus MAbs recognized markers can be identified and characterized individually.

Brief synopsis on the MAbs recognizing chicken cells of erythroid and monocytic lineages

Cell surface markers of normal or transformed hematopoietic cells in the human and murine systems have been extensively studied by using monoclonal antibody techniques. There are only a handful of MAbs which have been developed against chicken hematopoietic cells, and none of them are specific for embryonic precursor cells.

Hayman *et al*. (1982) developed a panel of MAbs against the temperature-sensitive mutant of ts34 AEV-transformed erythroblasts which had been grown at 41.5^{0}C for five days. Three MAbs were chosen for further characterization. MAb 4.2A5 recognized erythrocytes and AEV-transformed erythroblasts as well as granulocytes; MAb 4.5A5 reacted with erythroblasts and retrovirus-transformed producer cells, however, the authers did not address the specificity tests on other types of normal cells; MAb 4.6C1 was specific for erythroid cells at all stages.

Jurdic *et al*. (1982) produced a MAb, S1-37, from the fusion of spleen cells of a mouse immunized with AMV-transformed cells. S1-37 was shown to be specific for the

cells of the monocytic lineage. Its binding specificities revealed by radioimmunoassay and flow cytometric analysis were too low for further identification and characterization.

Miller *et al.* (1982) immunized mice with 1-day-old erythrocytes from inbred line 003 and hybrid strain Shaver-Starcross 288 and raised a MAb, MaEE1, against a 48Kd antigen which was expressed on the erythrocytes of 1-day-old peripheral blood and adult bone marrow. It was also present in the retina, muscle tissues, liver and on epithelia and lymphoid cells of young and adult chickens.

Sanders *et al.* (1982) were able to generate a MAb (190-4) against 1-day-old erythrocytes from the SC strain which recognized a 50Kd molecule expressed on the cell surface of erythrocytes, reticulocytes, chicken embryo cells and reticuloendotheliosis virus (REV)-transformed lymphoid cells, but not on the AEV-transformed erythroleukemia cells.

Kornfeld *et al.* (1983) obtained five different groups of MAbs by immunizing mice with normal macrophages and myeloid cells transformed by MC29, AMV and E26. Only one group was specific for myeloid lineage, predominantly reacting with immature myeloid cells. However, the authers failed to show the reactivities to normal cells except the macrophages.

Trembicki and Dietert (1985) produced 4 MAbs against 1-day cornell K-strain white leghorn chickens. MAb 10C6

detected a chicken fetal antigen (CFA) on 1-day-old chick erythrocytes. MAbs 3F12 and 4C2 recognized chicken adult antigen (CAA) on adult erythrocytes, whereas 9F9 reacted with all peripheral erythrocytes from both Japanese quail and chicken regardless of age.

Schmidt *et al.* (1986) immunized mice with plasma membranes from the ts34 AEV cell line HD3 induced to differentiate at 42^{0}C for five days. Only one out of eight groups of MAbs was specific for erythroid lineage, reacting only with reticulocytes.

The following chapters in this dissertation describe the first systematic attempt using MAbs in combination with other techniques to identify embryonic differentiation markers on normal avian hematopoietic cells and transformation-associated antigens on retrovirus-transformed ones.

CHAPTER 2

PRODUCTION OF MONOCLONAL ANTIBODIES AND THEIR CELL-TYPE SPECIFICITIES

Introduction

The lack of MAbs recognizing embryonic differentiation markers on avian hematopoietic precursor cells may be due to the use of inappropriate antigens for immunization. Because in most cases 1-day-old erythrocytes were chosen as antigens instead of embryonic cells, the MAbs developed were not specific for the embryonic precursor cells. Therefore it was decided to use different strategies for the immunizing protocols in order to produce MAbs which identify embryonic differentiation markers present on normal avian hematopoietic cells of the erythroid and monocytic lineages. Moreover the approaches used were designed to identify transformation-associated antigens in retrovirus transformed avian hematopoietic cells and to characterize the biochemical properties as well as to study the biological functions of these markers.

Several types of cells were used to immunize 6-week-old BALB/c BYJ female mice: 1) normal 3-day-embryo yolk sac cells, 2) normal 3-day-embryo megalocytestogether with AEV-

transformed nonproducer cells from 3-day-embryo yolk sac, 3) BM2 cells (AMV-transformed nonproducer cells from embryonic bone marrow), and 4) BM2/L cells (leukemogenic variant of BM2 cell line). Theoretically, 3-day-embryo yolk sac cells are rich in embryonic hematopoietic precursor cells, and the AMV/AEV nonproducer cells possess not only the transformation-associated antigens but also oncodevelopmental markers which are present normally only on the precursor cells. 10-14 days after each cell fusion, supernatants of hybridomas were screened against a panel of different types of cells as well as against yolk by indirect radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Only hybridomas showing continuous production of MAbs of potential interest were subcloned by the limiting dilution method. The isotypes of the MAbs were determined by the Ouchterlony (double diffusion) test using isotype specific antisera. MAbs were purified from culture media of the cloned hybridomas or from mouse ascites by using high-salt protein A-sepharose chromatography.

Material and Methods

Normal Cells

17-somite blastoderm. The blastoderm cells were obtained from the 17-somite stage at 2 days of incubation by mechanical dissociation with gentle pipetting and dispersed in α-MEM (GIBCO) containing 10% fetal bovine serum (FBS).

Cells were then filtered through a 1.5μ nylon mesh (Tetko Inc., New York) to remove any clumps (Moscovici *et al.*, 1983).

Yolk sac cells. The yolk sacs from 3rd or 4th day of embryogenesis were dissected free of other embryonic membranes, pooled and rinsed extensively with Tyrode's solution (8.0 g/L NaCl, 0.2 g/L KCl, 0.05 g/L $NaH_2PO_4 \cdot H_2O$, 1.0 g/L Glucose and 1.0 g/L $NaHCO_3$) to remove as much yolk as possible. The tissue were minced with scalpels and dispensed in α-MEM/10% FBS by gentle pipetting. The resulting cell suspension was then washed by centrifugation to remove residual yolk, after which the cells were resuspended in media and passed through nylon mesh to obtain a single cell suspension. The yolk sacs from 6th or 12th day of embryogenesis were first minced thoroughly with scalpels and then digested with 0.125% trypsin for 10-15 minutes at 37^0C (Moscovici *et al.*, 1975). The cell suspension was then washed by centrifugation and passed through nylon mesh as above.

Bone marrow cells. Bone marrow cells were flushed out of the tibias with BT-88 medium (GIBCO) containing 10% tryptose phosphate broth, 5% calf serum and 5% chicken serum by passing the cells through a syringe with a 22-gauge needle 3 times (Jurdic *et al.*, 1982). The cells were washed once, resuspended in medium and then filtered through nylon mesh.

Buffy coat. The buffy coat (WBC) from peripheral blood was harvested by Ficoll-Hypaque (Lymphocyte Separation Medium; Litton Bionetics) gradient centrifugation at 2,000 rpm for 20 minutes.

Macrophages. The buffy coat obtained from heparinized blood was seeded in BT-88 complete medium and 48 hours later the attached cells differentiated into macrophages. These cells were then detached from the petri dishes by adding the C-PEG solution (8.0 g/L NaCl, 0.29 g/L KCl, 0.2 g/L KH_2PO_4, 0.763 g/L Na_2HPO_4, 0.2 g/L EDTA, 3.7 g/L $NaHCO_3$ and 1.0 g/L Glucose, pH 8.0) for 5 minutes.

Chicken embryo fibroblasts (CEF). The fibroblasts from 10-day embryos were prepared according to the procedure described by Vogt (1969).

Transformed Cells

BM2/C3A cells. An AMV-transformed monoblastic nonproducer cell line, GM727, was generated by *in vitro* infection of 17-day-embryo bone marrow cells with AMV-B at a low multiplicity of infection (m.o.i. of 10^{-2} to 10^{-3}) (Moscovici & Moscovici, 1980). GM727 cells were then injected into 13-day embryos via the chorioallantoic vein. Four weeks after the injection, no overt case of leukemia was observed unless the chickens were challenged with helper viruses such as MAV-2 or RAV-7 (Moscovici *et al.*, 1982). However, the injected transformed cells could be retrived

from the bone marrow, and cloned by an *in vitro* colony assay. A cell line namely BM2/C3A was established. The cells in this line all express the v-*myb* proteins but are nonproducers and are nonleukemogenic.

BM2/L cells. The BM2/L cell line is a variant of BM2/C3A cell line. It was obtained from a BM2/C3A-injected bird which came down with leukemia involving liver, spleen and heart (Moscovici and Moscovici, unpublished results). The leukemic cells were reisolated and a new line was established, namely BM2/L, which when reinjected into chicken embryos induced a 90-100% incidence of leukemia.

6C2 cells. 6C2 is an AEV(RAV-2)-transformed erythroleukemia producer cell line obtained from infection of adult bone marrow cells *in vitro* (Beug *et al.*, 1982).

MSB 1 cells. MSB 1, obtained from U.S.D.A. (East Lansing, MI), is a lymphoblastoid producer cell line derived from a splenic lymphoma of chicken with Marek's Disease (Akiyama and Kato, 1974).

Viruses

AMV-B. The AMV subgroup B (AMV-B) was derived from standard laboratory stocks as described (Moscovici *et al.*, 1975).

AEV-A. The AEV subgroup A (AEV-A) is the ES-4 strain of AEV (RAV-1) originally obtained from Dr. J.M. Bishop (San Francisco, CA).

RAV-1 and RAV-2. The RAVs were prepared from our laboratory stocks.

Discontinuous Percoll Gradient

2 ml of cell suspension was layered on top of a discontinuous percoll gradient (20%/50%/70% for bone marrow cells and 30%/50%/70% for yolk sac cells) and centrifuged 15 minutes at 2,500 rpm. It has been demonstrated under these conditions that the 20%/50% interface from the bone marrow cells and the 30%/50% interface from the yolk sac cells are rich in the precursor cells and mononucleated cells, whereas the 50%/70% interface consists mostly of erythroblasts and other types of differentiated cells, and the cells from the pellet are terminally differentiated erythrocytes.

Hybridoma Production

Hybridomas were produced according to the protocol established in the Hybridoma Laboratory, Interdisplinary Center for Biotechnology Research, University of Florida. Spleen cells harvested from immunized mice were fused with SP2/O myeloma cells at a ratio of 7.5:1 (spleen cells : myeloma cells) using polyethylene glycol 1540. The supernatants of growth-positive hybridomas from 96-well flat bottom tissue culture plates were screened 12-14 days later by the indirect RIA and ELISA methods. Hybridomas which

produced monoclonal antibodies with potential interest were subcloned by the limiting dilution method.

Radioimmunoassay (RIA)

The cells used as targets in immunoassays were washed with PBS buffer containing 1% AHS (gamma globulin-free horse serum) and 0.02% sodium azide and resuspended to a final concentration of 20 x 10^6 cells/ml in PBS/azide/5% AHS. 50 μl of cell suspension and 100 μl of hybridoma supernatant was added to each well of 96-well flexible polyvinyl round-bottom microtiter plates (Dynatech) for 45 minutes at 4^0C. At the end of this incubation, plates were washed with PBS/azide/1% AHS and centrifuged at 1,100 rpm 3 times. 50 μl of ^{125}I-rabbit anti-mouse IgG (RaMIG), containing 1 x 10^5 cpm was then added and incubated with the cells for 45 minutes at 4^0C. Plates were washed with PBS/azide/AHS and centrifuged 3 times again. Individual wells were cut free from each plate with a hot-wire cutter, transferred into plastic tubes and counted in a gamma counter (LKB-Wallace Ria Gamma 1274, Pharmacia).

$$\text{Binding index} = \frac{\text{mean cpm bound with specific MAbs}}{\text{mean cpm bound with negative control MAb}}$$

Enzyme-Linked Immunosorbent Assay (ELISA)

96-well flat bottom immunoplates (Nunc, Denmark) were coated with 50 μl of yolk at a 1:40 dilution overnight and then blocked with 1X PBS containing 0.02% sodium azide and

1% BSA for 1 hour at room temperature. 100 μl of hybridoma supernatants were then incubated in wells for 45 minutes at room temperature followed by 100 μl alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) at a 1:1000 dilution in PBS/azide/BSA for 45 minutes at room temperature. The plates were finally incubated with 200 μl *p*-nitrophenyl phosphate (P-NPP) (1mg/ml) (Sigma) in pH 9.0 bicarbonate substrate buffer for 30 minutes to 2 hours in the dark and read on an ELISA reader (Molecular Devices; V Max). The plates were washed three times with PBS/azide/1% Tween-20 in between the steps.

Immunofluorescence staining

Live cells. The cells were incubated with 1 ml MAb supernatant for 30 minutes at 4^0C and washed before fixation with 30 μl 37% formaldehyde in 1 ml PBS for 20 minutes at 4^0C. The cells were washed again after fixation. 200 μl fluorescein-isothiocyanate (FITC) conjugated goat anti-mouse polyvalent IgG, A and M (FITC-GAM) at 1:50 dilution in PBS containing 1% normal goat serum was then added for 30 minutes at room temperature. After washing in PBS twice, the cell pellet was resuspended with 200 μl PPD-Glycerol/ PBS (1 ml 10X PBS, 3 ml deionized water, 6 ml glycerol and 1 to 2 flakes of p-phenylene diamine) and dropped onto slides to observe under the fluorescence microscope coverslipped.

Frozen sections. Chicken embryos from 3 and 6 days of incubation were dissected. Tissues of no more than a few mm thick were fixed in 4% paraformaldehyde fix (in 0.1 M sodium cacodylate buffer, pH 7.2-7.4) for 5 hours and shifted into 30% sucrose in 1X PBS overnight. Afterwards, the tissues were embedded in OCT compound (Lab-Tek; Miles) and frozen with liquid nitrogen. 10 μm Cryostat sections were then mounted onto slides which were precoated with Histostick (Accuraqe Biochemicals) and stored at -20^{0}C overnight prior to use. The slides were incubated with primary antibodies containing 0.3% triton-X in PBS for 30 minutes at room temperature. They were then washed in a PBS bath for 5 minutes and incubated with FITC-GAM/0.3% triton-X/1% normal goat serum for another 30 minutes at room temperature. The slides were again washed in PBS for 5 minutes followed by addition of a few drops of PPD-Glycerol/PBS and then examined under fluorescence microscope coverslipped.

Flow Cytometry

Cells to be examined were washed with 1X PBS/azide/AHS and incubated with 1 ml MAb supernatant for 1 hour on a rocker at 4^{0}C followed by incubation with 1:10 dilution of FITC-conjugated sheep anti-mouse IgG [F(ab')$_2$ fragment] (Sigma) for 30 minutes on a rocker at 4^{0}C. Cells were washed twice and resuspended in Hank's balanced salt solution (HBSS) containing 1% FBS at a concentration of 2 to

3×10^6 cells/ml. 1×10^4 cells were then analyzed on a FACStar-plus fluorescence-activated cell sorter (Becton-Dickinson, Mountain View, CA) by the parameters of forward light scatter and fluorescein fluorescence. Cellular excitation was obtained with an emission wavelength of 488 nm at an output power of 0.25W for fluorescein fluorescence. The FITC fluorescence emitted was filtered with a 530 nm long pass interface filter and a 530 band pass filter. The data were collected and analyzed by a Becton/Dickinson Consort 30 Computer program (Braylan *et al.*, 1982).

Immunoenzymatic Staining by APAAP (Alkaline Phosphatase and Monoclonal Anti-Alkaline Phosphatase) Complex

2×10^5 cells in 0.2 ml were cytofuged onto slides by Cytospin (Shandon Southern) at 300 rpm for 7 minutes. The slides were air-dried at room temperature for 2 hours followed by fixation with equal parts of acetone and methanol for 5 minutes at 4^0C. 1 ml MAb supernatants were added to the slides and incubated in a moist chamber for 30 minutes at room temperature. Anti-mouse immunoglobulins (DAKOPATTS; at 1:25 dilution) were then incubated for 30 minutes followed by APAAP complex (DAKOPATTS; at 1:50 dilution) for another 30 minutes. The slides were washed in a tris-buffered saline (TBS) bath for 1 minute in between the steps. Finally, alkaline phosphatase substrate was added onto the slides for 15-20 minutes and then washed off first with TBS and then with tap water (Cordell *et al.*,

1984). Slides were counter-stained with Giemsa stain at 1:20 dilution for 5 minutes.

Benzidine Staining

10 μl H_2O_2 (30%) was added to one ml of the benzidine solution (0.5 g benzidine in 100 ml 70% ethanol) immediately prior to use. Just a few drops of the staining mixture were deposited on the cell sample and left at room temperature in the dark for 5-10 minutes.

Induction of Cell Differentiation

BM2 cells. 1 x 10^7 BM2 cells were treated with 10 μg/ml lipopolysaccharide (LPS) and 0.25 μg/ml phorbol 12-myristate-13-acetate (PMA) or 2.5 μg/ml PMA alone in a 100-mm petri dish. 3 days later, most of BM2 cells had attached to the petri dish and had differentiated into macrophages.

6C2 cells. 6C2 cells were treated with 1.0 mM butyric acid for 3 days. Although 6C2 cells did not differentiate into mature erythrocytes with butyric acid, their proliferating potential had been arrested.

High-Salt Protein A-Sepharose Chromatography

Ascites (1:10 dilution) or hybridoma supernatants were adjusted to contain 1.5M glycine, 3M NaCl (pH 8.9), filtered through a 0.22 μ millipore filter and run through a protein

A-sepharose column (Sigma) twice to allow binding of IgG to the column. 5-10 column volumes of binding buffer (1.5M glycine, 3M NaCl, pH 8.9) was then percolated through the column to get rid of unbound proteins. Elution buffer (100 mM citric acid, pH 6.0) was then added to the column to elute the IgG, followed by regeneration buffer (100 mM citric acid, pH 3.0) to wash the column. Samples were collected by a fraction collector, neutralized to pH 7.0-7.2 with 1M tris buffer (pH 9.0) and read with a U.V. spectrophotometer at wavelength 280 nm. The concentration of IgG was calculated as: mg/ml = O.D. 280 nm / 1.4. Collected fractions were then dialyzed against 1x PBS/azide buffer overnight.

Results

Production, Subcloning and Isotyping of the MAbs

The rationale for MAb production is simple and straightforward, however, the goals turned out to be much tougher to achieve than we originally expected, especially from the fusions with spleens from mice immunized against 3-day-embryo yolk sac cells and megalocytes. Of nearly 2,500 hybridomas derived from 5 fusions, the predominant antibody specificities detected were for yolk components due to the fact that the unavoidably large amount of yolk was associated with the yolk sac cells. Only three MAbs exhibited potential interest, namely, 2E10, 3F6 and 1F7.

Fusions of spleens from mice immunized against BM2 cells and BM2/L cells yielded a panel of MAbs with various specificities against different types of hematopoietic cells rather than just MAbs specific for BM2 or BM2/L cells (Table 2-1). Three MAbs, 1H10, 2H1 and 3D7, from group VI, which displayed specificity for normal monocytic cells and AMV-transformed cells, were chosen for further studies.

These selected hybridomas were then subcloned by limiting dilution methods and isotyped by Ouchterlony double diffusion tests (Table 2-2). MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10 as well as 3F6-1E7 are all IgG1 (κ); only 1F7-1A3 is an IgM (κ).

Cell-Type Specificities of MAbs

As demonstrated by RIA, flow cytometry and immunofluorescence staining, MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 exhibited specificity for monocytic cells with a preferential reaction against BM2 lines. The RIA results also revealed a slight reaction of these MAbs with cells from the 20%/50% interface of a discontinuous percoll gradient from 2-week-old bone marrow cells (Table 2-3). Further analysis by flow cytometry (Figure 2-1) and APAAP immunoenzymatic staining (Figure 2-2) confirm that about 10%-20% of the cells are expressing different degrees of the differentiation markers recognized by this group of MAbs. Conversely, no expression of these markers was observed in

Table 2-1. The cell-type specificities of MAbs from BM2 and BM2/L fusions

Cell Type	Monoclonal Antibody Group					
	II	II	III	IV	V	VI[a]
Normal Cells						
Granulocytic	++[b]	+	-	-	+	-
Monocytic	++	++	+	+	++	+
Erythroid	++	+	+	-	-	-
Lymphoid	++	-	-	-	+	-
Transformed Cells						
AMV	++	++	++	++	++	++
AEV	++	++	+	+	-	-

[a]Only three MAbs from group VI were selected for further characterization because of their specificities for normal monocytic cells and AMV-transformed cells.
[b]++: RIA binding indexes >10.0; +: RIA binding indexes >5.0; -: RIA binding indexes <2.0.

Table 2-2. The isotypes of selected MAbs

MAb	Isotype
1H10-1F9	IgG1 (κ)
2H1-2A10	IgG1 (κ)
3D7-1C9	IgG1 (κ)
2E10-1E10	IgG1 (κ)
3F6-1E7	IgG1 (κ)
1F7-1A3	IgM (κ)

Table 2-3. RIA binding indexes of MAbs to different cell types

Cells[a]	Monoclonal Antibody 1H10-1F9	2H1-2A10	3D7-1C9	2E10-1E10	3F6-1E7	1F7-1A3
TRANSFORMED						
6C2	1.19[b]	1.21	1.66	26.35	3.03	3.96
3DYS-AEV	N.T.	N.T.	1.00	6.48	5.38	3.04
6DYS-AEV	N.T.	N.T.	N.T.	25.94	12.52	5.27
MSB1	1.26	0.59	0.34	14.06	7.78	5.17
BM2/C3A	43.55	38.80	38.01	9.91	9.61	1.25
BM2/REC1	34.09	50.83	57.77	8.03	N.T.	N.T.
BM2L/A1	29.98	35.05	41.72	8.26	9.72	1.29
NORMAL						
Mϕ	4.89	6.85	5.87	N.T.	N.T.	N.T.
RBC(PB)	0.77	1.52	1.14	2.58	1.09	1.31
WBC(PB)	1.15	2.25	1.63	2.24	1.25	1.11
CEF	0.83	0.97	0.88	0.87	0.75	N.T.
2DYS	0.52	0.94	1.13	2.34	2.01	N.D.
3DYS	1.52	1.40	1.16	2.18	2.11	5.72
6DYS	0.68	1.60	0.84	3.27	1.12	N.T.
12DYS	0.69	1.28	0.62	7.59	0.52	N.T.
BM 20/50	2.88	3.48	2.89	4.76	1.98	N.T.
BM 50/70	1.62	2.39	1.91	7.49	1.63	N.T.

[a]6C2, AEV-transformed producer cell line from adult bone marrow; 3DYS-AEV, AEV-transformed 3-day-embryo yolk sac cells; MSB1, Marek's Disease Virus-transformed lymphoblastoid producer cell line; BM2, AMV-transformed non-producer cell line from embryonic bone marrow; BM2/C3A, a subclone of BM2; BM2/REC1, a subclone recovered from the bone marrow of BM2/C3A injected chick; BM2L/A1, a leukemogenic variant of BM2/C3A; Mϕ, macrophage; PB, peripheral blood; CEF, chicken embryo fibroblast; BM 20/50, 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells; N.T., not tested.

[b]RIA binding indexes: see Materials and Methods for details.

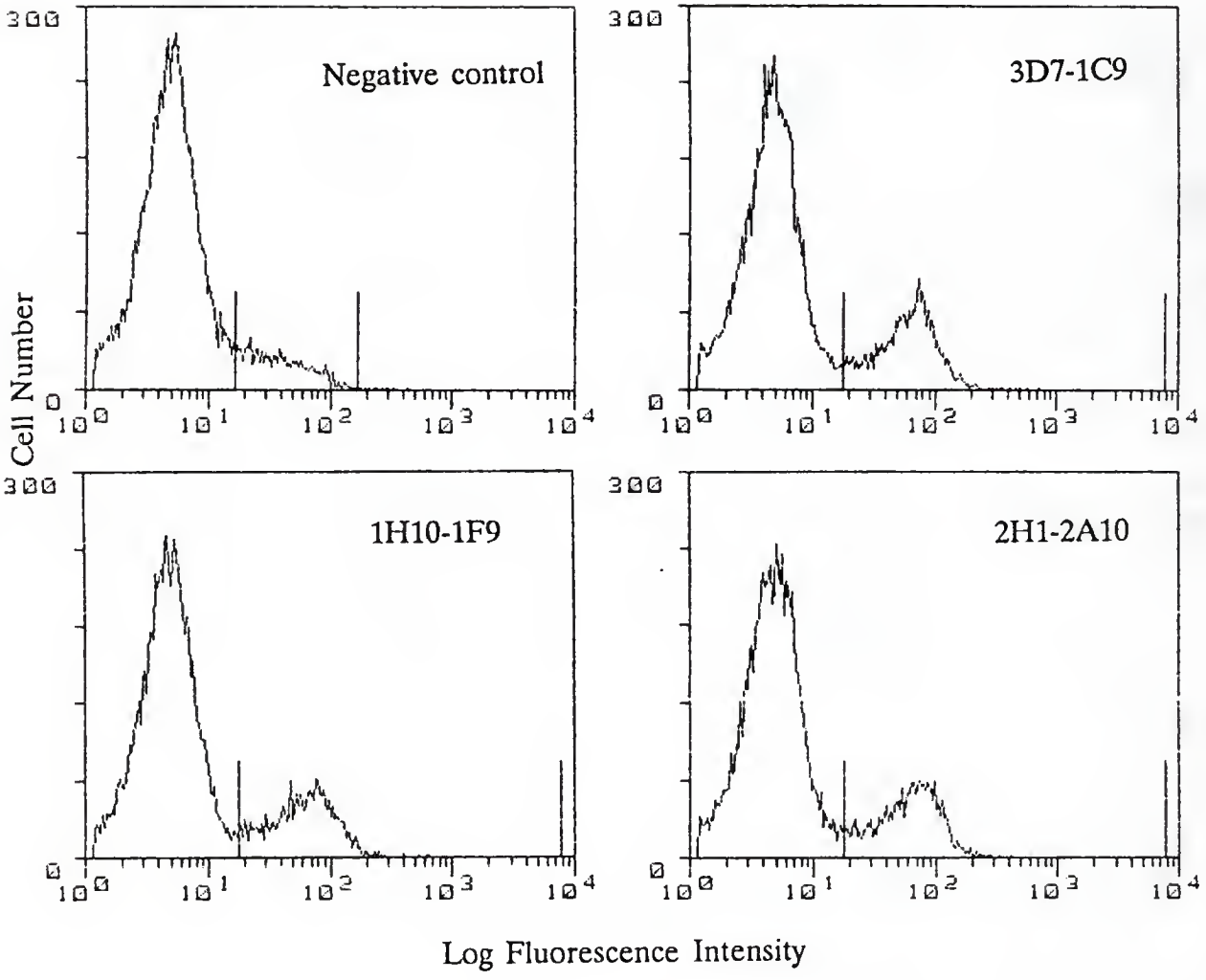


Figure 2-1. Flow cytometric analysis of the 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells. The cells were labeled with negative control MAb, 1H10-1F9, 2H1-2A10 and 3D7-1C9 respectively. The background fluorescence resulting either from nonspecific binding or autofluorescence was present in 10% of the cells. 10-20% of the cells were positive for MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9.

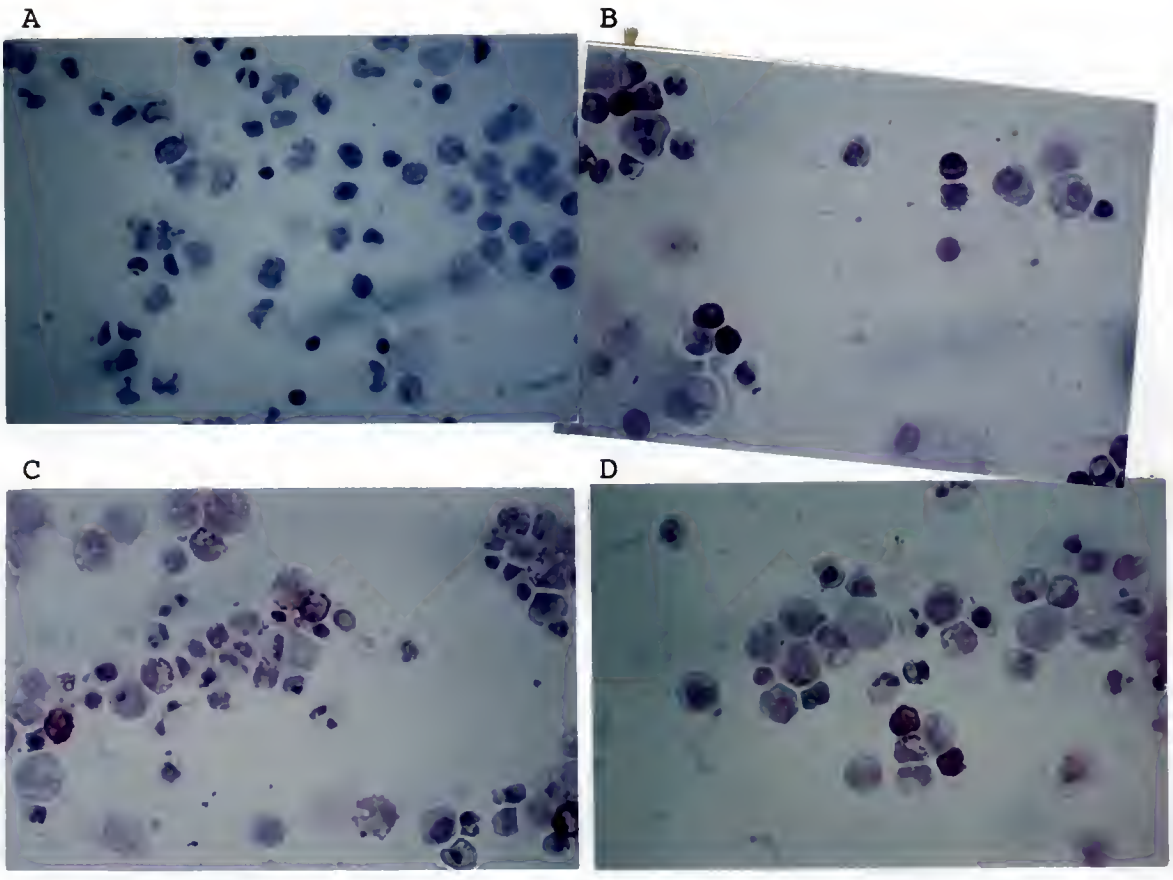


Figure 2-2. APAAP immunostaining of 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells (600x). The cytofuges were treated with (A) negative control MAb, (B) 1H10-1F9, (C) 2H1-2A10 and (D) 3D7-1C9 respectively. Positive cells were labeled with purple-reddish products around the cell surface and/or inside the cytoplasm.

the cells from the 50%/70% interface (Table 2-3 and Figure 2-3).

1F7-1A3 recognizes the AEV-transformed yolk sac cells, MSB1 cells and 20% of the cells in the 30%/50% interface of a discontinuous percoll gradient of yolk sac cells (Table 2-3 and Figure 2-4). Interestingly enough, its reaction to 6C2 cells is amplified after the treatment with neuraminidase (discussed in Chapter 4 in more detail).

3F6-1E7 detects a differentiation marker present mainly on AEV-transformed yolk sac cells, BM2 cell lines and MSB1 cells as well as 10% of cells from the 30%/50% interface of a percoll gradient of normal yolk sac cells (Table 2-3 and Figure 2-4).

2E10-1E10 possesses a variety of cell-type specificities such as 6C2 cells, AEV-transformed yolk sac cells, MSB1 cells, BM2 cells, 6 & 12-day-embryo yolk sac cells and 2-week-old bone marrow cells (Table 2-3, Figure 2-5 & Figure 2-6). However, there is no reaction to the terminally differentiated hematopoietic cells.

The cell-type specificities of MAbs are summarized in Table 2-4.

Induction of Cell Differentiation

Treatment of BM2/C3A cells with 0.25 μg/ml PMA and 10 μg/ml LPS or 2.5 μg/ml PMA alone for 3 days caused the BM2/C3A cells to attach to the petri dishes and become

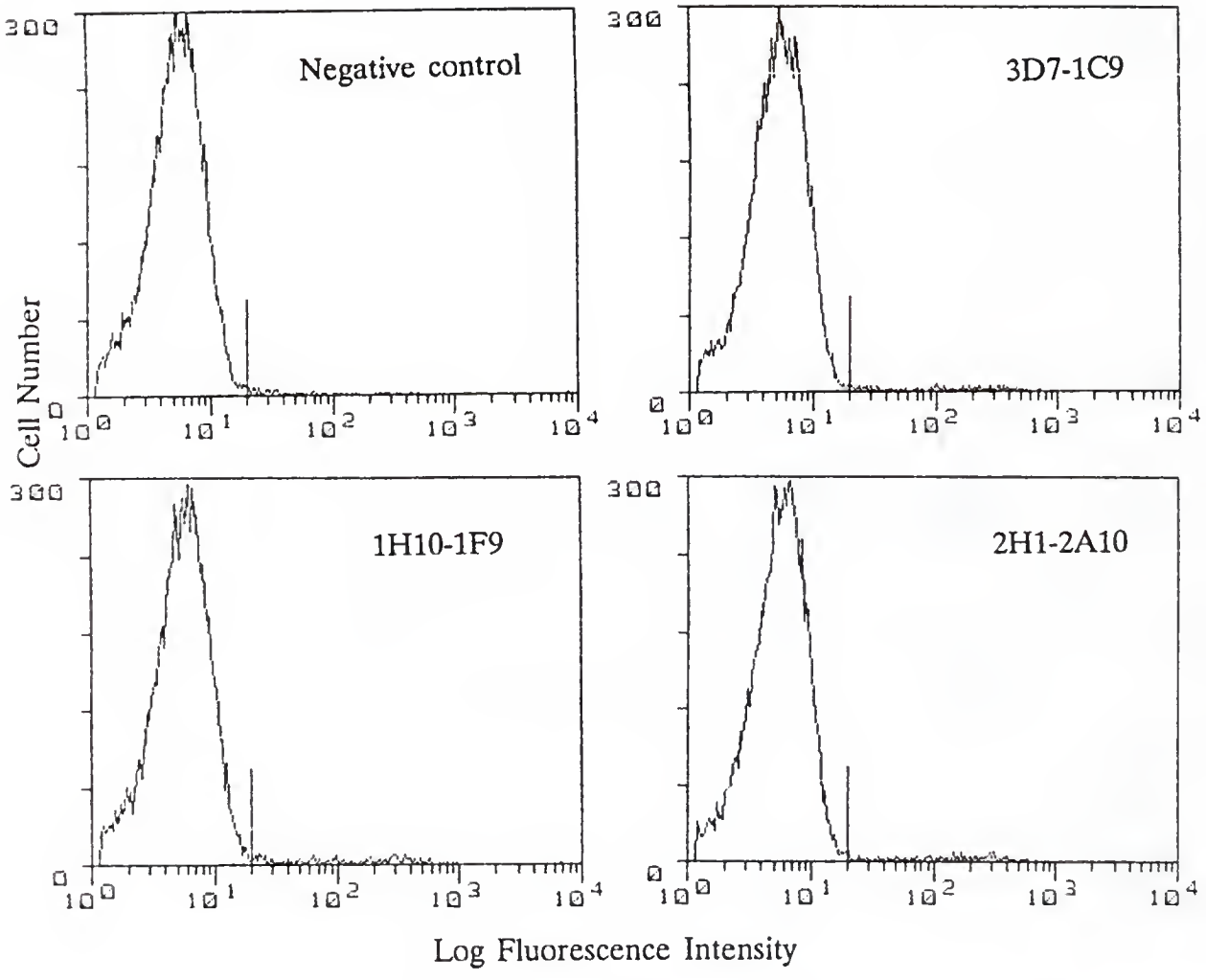


Figure 2-3. Flow cytometric analysis of the 50%/70% interface of a percoll gradient of 2-week-old bone marrow cells. The cells were labeled with negative control MAb, 1H10-1F9, 2H1-2A10 and 3D7-1C9 respectively. The results showed no reaction of MAbs with these cells at all.

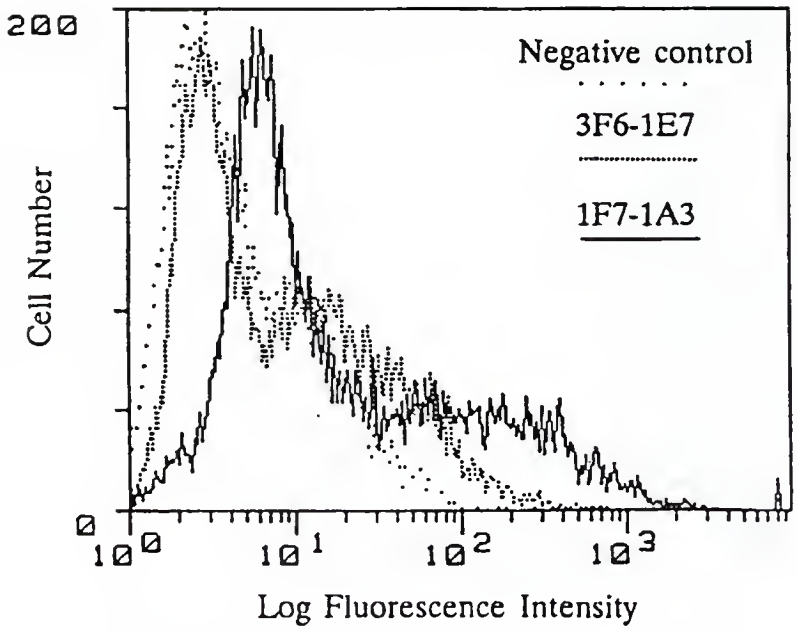


Figure 2-4. Flow cytometric analysis of the 30%/50% interface of a percoll gradient of 4-day-embryo yolk sac cells. Cells were tagged with negative control MAb, 1F7-1A3 and 3F6-1E7 respectively. The analysis showed that 3F6-1E7 label 10% of the cells, whereas 20% of the cells are positive for 1F7-1A3.

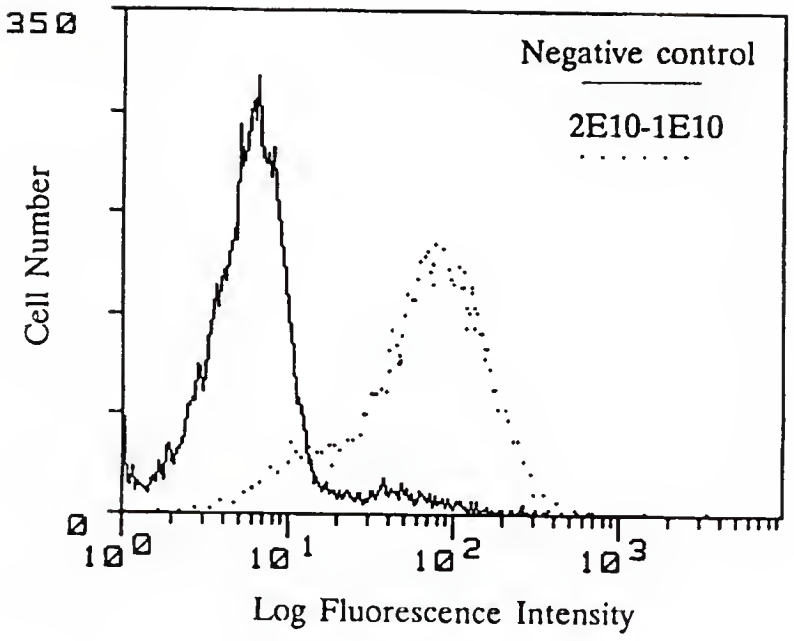


Figure 2-5. Flow cytometric analysis of the 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells tagged with MAb 2E10-1E10. Almost 80% of the cells were positive for MAb 2E10-1E10.

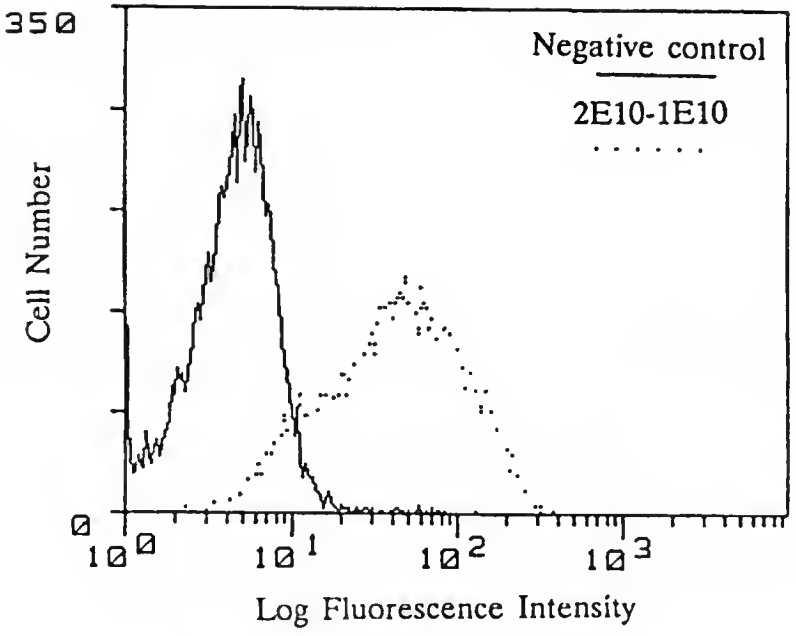


Figure 2-6. Flow cytometric analysis of the 50%/70% interface of a percoll gradient of 2-week-old bone marrow cells tagged with MAb 2E10-1E10. 2E10-1E10 recognized nearly 70% of the cells.

Table 2-4. Summary of the cell-type specificities of MAbs

Groups	MAbs	Specificities
I	3D7-1C9 2H1-2A10 1H10-1F9	BM2 cell lines Normal monocytic cells
II	3F6-1E7	AEV-transformed yolk sac cells BM2 cell lines MSB1 (Marek's Disease) 10% of cells from the 30%/50% interface of a percoll fraction of 3-day-embryo yolk sac cells
III	1F7-1A3	AEV-transformed yolk sac cells MSB1 (Marek's Disease) 20% of cells from the 30%/50% interface of a percoll fraction of 3-day-embryo yolk sac cells
IV	2E10-1E10	6C2 cells AEV-transformed yolk sac cells MSB1 (Marek's Disease) BM2 cell lines 6-day-embryo yolk sac cells 12-day-embryo yolk sac cells 2-week-old bone marrow cells

differentiated into macrophage-like cells. In addition, their proliferating potential (Table 2-5) and ability to bind MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 were dramatically decreased (Figure 2-7). Therefore, the differentiation markers recognized by these MAbs may be candidates for transformation-associated antigens in this system.

Although 6C2 cells were not induced to terminally differentiate into erythrocytes with the treatment of 1mM butyric acid for 3 days, their proliferating potential had been impaired (Table 2-6) and the expression of the 2E10-1E10-recognized marker was also reduced (Figure 2-8).

Reactivity to the Envelope Proteins of Retroviruses

The RAV-1 or RAV-2 infected CEF cells were used as control to exclude the possibility that MAbs might react with the envelope proteins of retroviruses which were expressed on the cell surface. 3F6-1E7 and 2E10-1E10 were shown to have no reaction to either CEF cells or RAV-infected CEF cells (Table 2-7).

Discussion

Four groups of MAbs were selected among nearly 5,000 hybridoma supernatants from ten fusions. Characterization of their cell-type specificities was achived by RIA, immunofluorescence staining, flow cytometry and immunoenzymatic staining. 1) MAbs 1H10-1F9, 2H10-2A10 and

Table 2-5. Comparison of the proliferating potential of BM2/C3A cells and 2.5μg/ml PMA-diffentiated BM2/C3A cells

	BM2/C3A[b]	BM2/C3A + PMA[b]
Number of cells after 3 days of incubation[a]	58×10^6	6×10^6

[a] 10×10^6 cells were seeded per 100-mm petri dish.
[b] The number of cells was expressed as the average from 6 dishes.

Figure 2-7. Reactivities of MAbs with BM2 cells and differentiated BM2 cells. BM2 cells were differentiated into macrophage-like cells by the treatment of 0.25 μg/ml PMA and 10 μg/ml LPS for three days. The reactivities are expressed as the MAb binding indexes as determined by RIA.

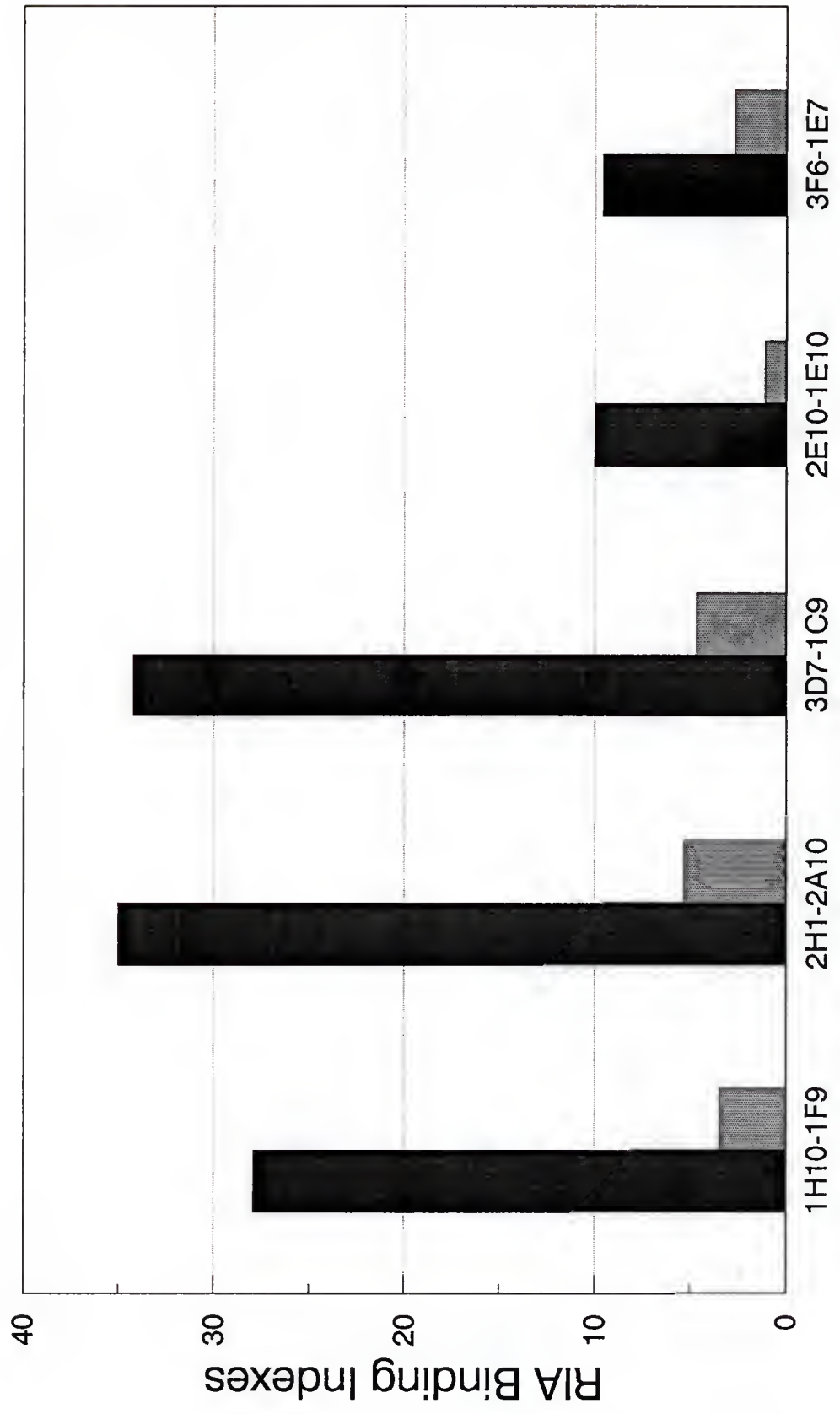

RIA Binding Indexes
0
10
20
30
40
1H10-1F9
2H1-2A10
3D7-1C9
2E10-1E10
3F6-1E7
Control
PMA & LPS

Table 2-6. Comparison of the proliferating potential of 6C2 cells and 1mM butyric acid-treated 6C2 cells

	6C2[b]	6C2 + Butyric acid[b]
Number of cells after 3 days of incubation[a]	40×10^6	11.5×10^6

[a] 10×10^6 cells were seeded in 60-mm petri dish.
[b] The number of cells was expressed as the average from 6 dishes.

Figure 2-8. Reactivities of MAbs with 6C2 cells and butyric acid-treated 6C2 cells. 6C2 cells were treated with 1.0mM butyric acid for three days. Though 6C2 cells were not differentiated into erythrocytes, the proliferating potential had been impaired. The reactivities are expressed as MAb the binding indexes as determined by RIA.

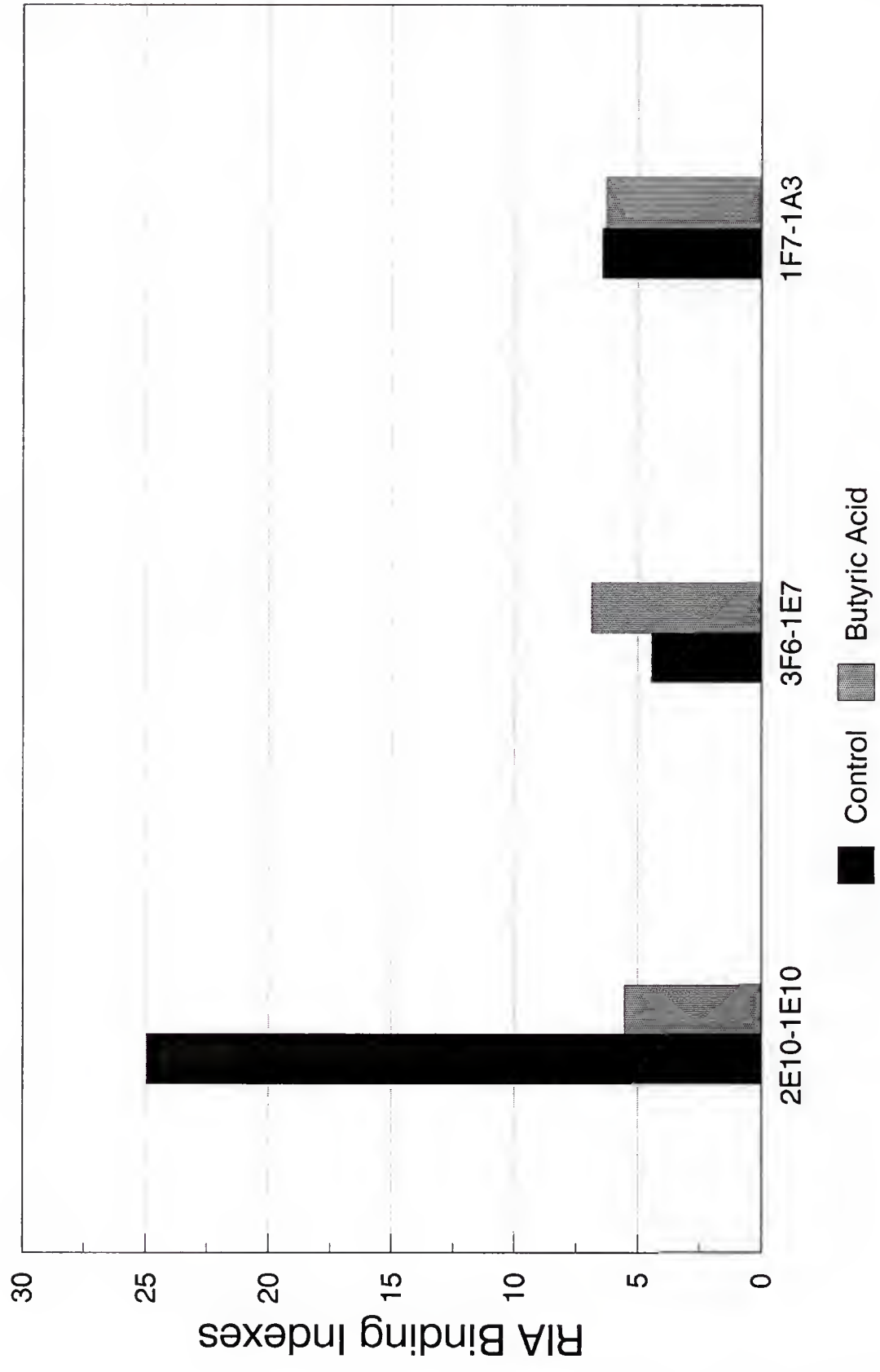

RIA Binding Indexes
30
25
20
15
10
5
0
2E10-1E10
3F6-1E7
1F7-1A3
Control
Butyric Acid

Table 2-7. RIA binding indexes of MAbs to normal CEF and RAV-infected CEF

Cells	Monoclonal Antibody	
	2E10-1E10	3F6-1E7
CEF	0.87	0.75
CEF (RAV-1)	1.08	0.75
CEF (RAV-2)	1.10	0.80

3D7-1C9 are specific for transformation-associated antigens present preferentially on BM2 cell lines rather than on normal monocytic cells. 2) MAb 1F7-1A3 recognizes AEV-transformed yolk sac cells, MSB1 cells and 20% of cells from the 30%/50% interface of a discontinous percoll gradient of 4-day-embryo yolk sac cells. 3) MAb 3F6-1E7 reacts with AEV-transformed yolk sac cells, BM2 cells and MSB1 cells as well as 10% of cells from the interface of a discontinous percoll gradient of 4-day-embryo yolk sac cells. 4) MAb 2E10-1E10 defines a marker present on different tumor cells, yolk sac cells and bone marrow cells instead of terminally differentiated cells.

The expression of transformation-associated antigens recognized by MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 is possibly up-regulated by v-*myb* proteins, but is diminished after the BM2 cells are differentiated by PMA. Future investigators may want to determine 1) whether the expression of these antigens is regulated at the transcriptional and/or the translational level or is due to posttranslational modification, such as aberrant glycosylation and/or sialylation, and 2) whether the expression of these markers is essential for the transforming process or if they are merely the by-products of transformation.

Although 2E10-1E10 displays a wide range of cell-type specificities, it labels primarily 6C2 cells and

AEV-transformed 6-day-embryo yolk sac cells and it does not recognize terminally differentiated cells such as erythrocytes, macrophages, lymphocytes and PMA-differentiated BM2/C3A cells. These results lead us to speculate that 2E10-1E10 may react only with proliferating hematopoietic cells. This possibility was supported by the observation that the binding specificities of 2E10-1E10 to 6C2 cells was diminished after treatment with butyric acid which inhibited the proliferating potential of 6C2 cells without the induction of differentiation. Moreover, this marker starts appearing at the 4th day of embryogenesis (discussed in Chapter 3 in more detail) and its expression is enhanced after the cells are transformed by AEV.

LPS only induces the BM2 cells to attach to the petri dishes but does not induce differentiation. As a matter of fact, PMA alone can induce both the attachment and differentiation of the BM2 cells even at a low concentration of 0.25 μg/ml. The reason why we added 10 μg/ml LPS to the PMA treatment was simply because we didn't want to see "any fish sneak out of the net", as the Chinese proverb says.

Butyric acid has been shown to induce the differentiation of the AEV strain R (RAV-2)-transformed erythroleukemia cells from SC strain chickens (Nelson *et al.*, 1982), but was incapable of differentiating 6C2 cells even though their proliferating potential had been apparently impaired. This is possibly due to the

difference in the susceptibilities to butyric acid of various avian erythroleukemia cell lines and/or sublines.

Immunoperoxidase staining procedures were not used because significant amounts of endogenous peroxidase present in granulocytes, erythroid cells and macrophages would give rise to unwanted background staining, and it is rather difficult to abolish this activity by exposing samples to peroxidase inhibitors (such as H_2O_2 and methanol) without causing antigenic denaturation (Cordell *et al.*, 1984). In contrast endogenous alkaline phosphatase activity survives poorly in cytofuge slide preparation and any residual activity may be selectively inhibited by including levamisole in the enzyme substrate solution (Ponder and Wilkinson, 1981). Therefore the APAAP immunostaining technique was chosen for our studies. Wet slides without any mounting fluid were then photographed becaused it was observed that the nonaqueous mounting solution dissolved the reaction product, whereas an aqueous mounting medium will disrupt the Giemsa counter-stain.

The antigens recognized by MAbs 1F7-1A3, 2E10-1E10 and 3F6-1E7 could only be detected by RIA and immunofluorescence staining and not by APAAP immunostaining technique. It is very likely that these markers may be so fragile that they were destroyed during the fixation (acetone and ethanol) and/or any subsequent steps.

CHAPTER 3

IDENTIFICATION OF TARGET CELLS FOR MABS IN THE BONE MARROW AND YOLK SAC

Introduction

The avian hematopoietic precursor cells represent only a small population in the blood-forming organs, however, it is this small population that builds up the entire hematopoietic repertoire of various types of specialized blood cells with different functions. The nature of self-renewal and commitment remains an enigma, but if the precursor cells can be isolated from the heterogenous population of hematopoietic cells for direct studies, it would provide us with invaluable pieces of information to solve the enigma. However, these cells are morphologically unrecognizable, and until now no specific markers had been identified to facilitate the purification of these cells. One of the specific aims of this project was to develop MAbs against differentiation markers specifically present on the surface membranes of the avian hematopoietic precursor cells. These cells have been purified and enriched by using FACS or immunomagnetic beads as follows. Bone marrow cells or yolk sac cells are treated with specific MAbs followed by FITC-conjugated or magnetic bead coated with secondary

antibodies. Fluorescence-positive and -negative populations are then separated by the FACS, whereas the magnetic bead-bound cells are separated from the negative population by a magnetic field (negative selection). The separated fractions are then identified by indirect methods, such as AMV/AEV transforming assays and BFU-E/CFU-E colony forming assays. For example, if MAb-positive fractions produce colonies derived from the CFU-E instead of the BFU-E, the antigen must be expressed later than the BFU-E stage; if it produces AMV-transformed colonies, but neither AEV-transformed colonies nor BFU-E/CFU-E colonies are developed, the MAb ought to be specific for the target cells for AMV, *i*.e., cells of the monocytic lineage.

These approaches helped us to confirm the cell-type specificities of the MAbs that we developed.

Materials And Methods

FACS Sorting

After being analyzed on a FACStar-plus sorter, according to the procedure described in the previous chapter, the cells were sorted into positive and negative fractions at a rate of 1,000 cells/sec.

Immumomagnetic Beads Separation

10 x 10*6* Cells were tagged with 1 ml of MAb supernatants for 1 hour at 4^{0}C on a rocker and then washed

with PBS/azide/1% AHS followed by incubation with magnetic beads coated with sheep anti-mouse IgG, (Fc) (Dynabeads M-450; Dynal Inc.) or magnetic particles coated with goat anti-mouse IgM (Advanced Magnetics) at a bead to cell ratio of 4 to 1 for 30 minutes at 4^0C on a rocker. Cells bound to the beads were separated from the negative population that remained in suspension by applying a cobalt steel magnetic force (Dynal MPC-1; Dynal Inc.) for 1 minute (Cruikshank *et al.*, 1987). This process was repeated twice. It was not possible to separate viable cells (positive selection) from the magnetic beads because of technical limitations.

Retrovirus Transforming Assays

The transforming assays were performed according to the protocol described by Moscovici *et al.* (1983). Briefly, cells were infected with retroviruses at high m.o.i. The virus adsorption was carried out 30 minutes at 4^0C and then 30 minutes at room temperature. 1 x10^5 AEV-infected cells was seeded in 2 ml semi-solid α-medium containing 20% FBS, 10% ACS, 1% BPA, 0.1% 10^{-1}M β-mercaptoethanol, 0.1% gentamycin and 25% methocellulose per 35-mm dish. Whereas, 1 x10^5 AMV-infected cells was mixed with 1 ml F12 overlay medium containing 20% 2X F12, 6% calf serum, 2% chicken serum, 10% tryptose phosphate, 1% 100X vitamins, 1% 100X folic acid and 40% fibroblast-conditioned medium as well as 20% of 1.8% Bacto agar, and then overlayed on top of 2 ml

3.6% hard agar base in 35-mm petri dishes. The transformed colonies were scored after 6-12 days of incubation.

BFU-E/CFU-E Colony Assays

0.5 ml of cell suspension were mixed with α-medium containing 20% FBS, 10% ACS, 1% BPA, 0.1% 10^{-1}M β-mercaptoethanol, 0.1% gentamycin and 25% methocellulose and seeded at $1x10^5$ cells per 35-mm dish. The CFU-E were scored after 3-4 days of incubation, whereas BFU-E were scored after 6-7 days of incubation (Samarut and Bouabdelli, 1980).

Results

FACS Sorting

In order to identify the target cells for the MAbs in the bone marrow, FACS was utilized to separate the MAb-positive population and MAb-negative population. The bone marrow cells were analyzed before and after the FACS sorting (Figure 3-1 and 3-2). Transforming assays and colony-forming assays were performed on both negative and positive populations.

The results obtained in the transforming assays and colony-forming assays (Table 3-1) confirm that the 3D7-1C9 positive population (10%-20% of the cells from the 20%/50% interface of a discontinuous percoll gradient of 2-week-old bone marrow cells) represents the target cells for AMV, *i*.e., cells of the monocytic lineage.

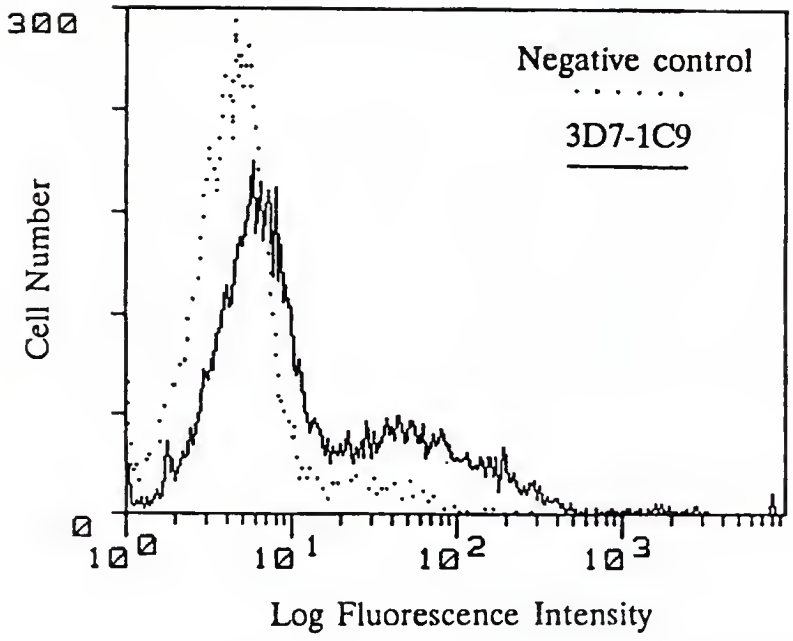


Figure 3-1. Flow cytometric analysis of 3D7-1C9-tagged bone marrow cells before sorting. 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells were tagged with 3D7-1C9 MAb. The analysis showed that 10-20% of the cells are positive for MAb 3D7-1C9.

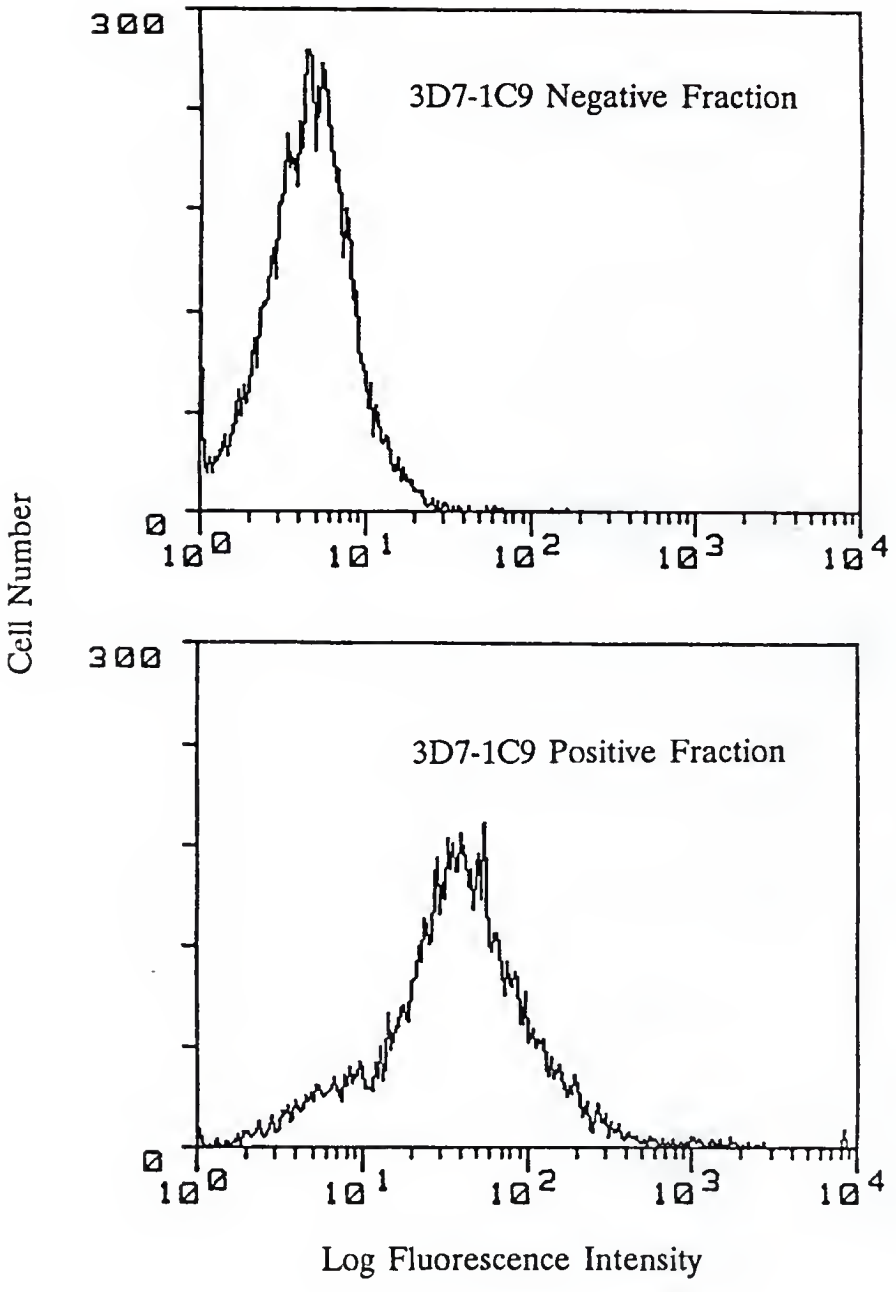


Figure 3-2. Flow cytometric analysis of 3D7-1C9-tagged bone marrow cells after sorting. 20%/50% interface of a percoll gradient of 2-week-old bone marrow cells were sorted into 3D7-1C9 positive fraction and 3D7-1C9 negative fraction followed by the FACS analysis. The 3D7-1C9 positive fraction still contained about 20% negative cells due to contamination during the sorting.

Table 3-1. Characterization of the target cells isolated with MAb 3D7-1C9 from the bone marrow

Colonies ($1x10^5$ cells)	3D7-1C9 positive population	3D7-1C9 negative population
CFU-E	34.0 ± 5.0	1620.5 ± 52.5
BFU-E	12.0 ± 3.0	512.0 ± 132.0
AEV-A	26.0 ± 2.0	144.0 ± 7.0
AMV-B	592.5 ± 20.5	86.5 ± 13.5

20%/50% interface of a percoll gradient of 2-week-old bone marrow cells were tagged with 3D7-1C9 and then sorted by FACStar followed by transforming assays and colony-forming assays. The results show that 3D7-1C9 positive population contain the target cells for AMV, *i.e.*, cells of monocytic lineage, rather than BFU-E/CFU-E or the target cells for AEV.

On the other hand, the results of colony-forming assays and retrovirus transforming assays suggest that MAb 2E10-1E10 not only recognize the target cells for AEV and AMV but also the BFU-E and CFU-E (Table 3-2). This finding prompted us to suggest in Chapter 2 that MAb 2E10-1E10 react with proliferating hematopoietic cells.

Immunomagnetic Bead Separation

Cells from 30%/50% interface of a discontinuous percoll gradient of 4-day-embryo yolk sacs were incubated with MAbs 3F6-1E7 or 1F7-1A3 followed by magnetic beads conjugated with secondary antibodies. Only the negatively selected population of MAbs were used for the assays. There was about 60% reduction in the BFU-E and the CFU-E colonies from 1F7-1A3 negative population. However, no significant difference in transformed colonies was found (Table 3-3). In other words, MAb 1F7-1A3 recognizes erythroid cells at the BFU-E and the CFU-E stages but not the pre-BFU-E stage, since it does not recognize cells at the pre-BFU-E stage which are the target cell for AEV in the yolk sac. Conversely MAb 3F6-1E7 seems not to react with either the BFU-E/CFU-E or the target cells for the retroviruses. It probably recognizes a differentiation marker expressed on the stem cell and precursor cell populations at an earlier stage than pre-BFU-E one.

Table 3-2. Characterization of the target cells isolated with MAb 2E10-1E10 from the bone marrow

Colonies ($1x10^5$ cells)	2E10-1E10 positive population	2E10-1E10 negative population
CFU-E	474.0 ± 27.0	0.0 ± 0.0
BFU-E	282.0 ± 2.0	0.0 ± 0.0
AEV-A	59.0 ± 1.0	0.5 ± 0.5
AMV-B	403.0 ± 3.0	5.0 ± 2.0

20%/50% interface of a percoll gradient of 2-week-old bone marrow cells were tagged with 2E10-1E10 and then sorted by FACStar followed by transforming assays and colony-forming assays. The results reveal that MAb 2E10-1E10 recognize not only BFU-E/CFU-E, but also the target cells for AEV and AMV.

Table 3-3. Characterization of the target cells isolated with MAbs from the yolk sac

Colonies (1×10^5 Cells)	Monoclonal Antibody			
	Control	3F6-1E7	2E10-1E10	1F7-1A3
CFU-E	3313.1±142.5	2576.2±232.4	1597.0±78.4	1487.5±103.4
BFU-E	1211.9± 11.9	899.2± 67.3	684.8±73.3	501.1± 23.8
AEV-A	135.6± 6.4	115.0± 7.0	108.1± 5.2	102.0± 11.0
AMV-B	50.0± 5.0	46.0± 2.0	36.8± 2.4	44.0± 3.0

30%/50% interface of a percoll gradient of 4-day-embryo yolk sac cells were treated respectively with MAbs 1F7-1A3, 2E10-1E10 or 3F6-1E7 followed by immunomagnetic bead separation. The MAb-negative populations were collected to perform colony-forming assays and transforming assays. See text for more details.

The MAb 2E10-1E10 negative population in the 4-day-embryo yolk sac cells exhibited nearly 50% reduction in the BFU-E/CFU-E colonies and 30% in the AMV-transformed colonies repectively (Table 3-3). Moreover, I have shown in Chapter 2 that MAb 2E10-1E10 has no binding specificities for 2- or 3-day-embryo yolk sac cell. These results indicate that the marker identified by MAb 2E10-1E10 starts appearing after the 4th day of embryogenesis.

Discussion

The FACS sorting and immunomagnetic bead techniques allow us to perform direct studies on MAb-positive and/or MAb-negative populations. The results from colony-forming assays and transforming assays showed that MAb 3D7-1C9 can purify the target cells for AMV, *i.e.*, cells of the monocytic lineage, MAb 2E10-1E10 define a marker present on proliferating hematopoietic cells and it starts appearing only after the 4th day of embryogenesis and that MAb 1F7-1A3 recognize erythroid cells at BFU-E and CFU-E stages.

MAb 3F6-1E7 recognizes only some tumor cell lines and 10% of cells from the 30%/50% interface of a discontinuous percoll gradient of 3- and 4-day-embryo yolk sac cells, and it does not possess the specificities for the terminally differentiated cells and the lineage-committed progenitor cells. However there is no direct evidence yet to support the idea that the 10% of the cells recognized by MAb 3F6-1E7

represent the stem cell and precursor cell populations in the yolk sac. In order to prove this, there are two obstacles that need to be overcome. One is to improve the FACS sorting condition to prevent yolk sac cells from lysing. The other is to establish a long-term culture of normal avian yolk sac cells. Once these problems are solved, it will become possible to obtain the long-term culture of the FACS-sorted 3F6-1E7 positive cells and then we can compare the results from the transforming assays and colony-forming assays between the 3F6-1E7 positive population with and without the long-term culture. Theoretically, in the long-term culture, the stem cell and precursor cell populations will proliferate and undergo the normal differentiation program to become the committed progenitor cells and mature cells. If the 3F6-1E7 positive cells indeed represent these populations, the increased number of retrovirus-transformed colonies and BFU-E/CFU-E colonies will then be observed in the 3F6-1E7 positive cells with long-term culture. Ultimately, 3F6-1E7 positive cells should be capable of repopulating the bone marrow of irradiated chicks.

It is still a puzzle why MAb 1F7-1A3 reacts with lymphoblastoid cell line-MSB1. Maybe the marker recognized by 1F7-1A3, which is normally present on the embryonic BFU-E/CFU-E only, can be regarded as an onco-fetal antigen, *i*.e., its expression being turned on in the AEV-transformed

yolk sac cells as well as in the MSB1 cells instead of in other types of transformed cells. This interesting finding also implies that the relationship between the erythroid and lymphoid lineage may be closer than we originally thought.

The bone marrow cells were sorted into fluorescence-positive and -negative populations using the FACStar-plus at a rate of 1,000 cells/sec. At this rate it took approximately 2-3 hours to collect the minimum workable number of cells, *i.e.*, $5x10^5$ MAb-positive cells which account for 10-20% of cells from the 20%/50% interface of a discontinuous percoll gradient of 2-week-old bone marrow cells. The results of flow cytometry, retrovirus transforming assays and colony-forming assays showed that there is a slight contamination of MAb-negative cells in the positive population. To increase the purity of the collection by reducing the analysis rate or by processing a "two-run" procedure would be too time-consuming and the viability or the behavior of the sorted cells might be influenced.

The yolk sac cells are extremely delicate and fragile compared to other types of cells. Suffering from the "abuse" of scalpel mincing, extensive washing, percoll fractionation, as well as FACS sorting, the cell membranes could have been damaged in such a way that only less than 10% of the sorted cells stayed intact, while the rest were all lysed. We have tried different approaches such as

changing the sheath fluid, minimizing the laser power and electric charge, reducing the centrifugation speed and changing the collecting tubes etc. None so far gave us satisfactory results.

Nevertheless, this is the pioneer study of the avian hematopoietic system by FACS analysis. Once all the conditions are standardized, it will become a tremendously powerful tool to identify the stem cell and precursor cell populations of the avian hematopoietic system, especially in the yolk sac.

There are two disadvantages in using the immunomagnetic beads for cell separation. First, its sensitivity is much lower than that of the FACS sorting, *i.e.*, it yields less pure separation than FACS does. Second, because of the strength of positive cells binding to magnetic beads, trypsinization is needed to free the magnetic beads from the cell surface. Cell surface receptors for retroviruses and growth factors will be destroyed by the trypsinization, not to mention the possibility that the yolk sac cells may become more susceptible to lysis. As a result, few viable cells remain for further study. Nevertheless, compared to the FACS sorting, immunomagnetic bead separation is not as time-consuming and has the advantage of not damaging the yolk sac cells. Therefore, immunomagnetic beads separation represents the most useful technique so far to study the influence of MAbs in the yolk sac system.

CHAPTER 4

BIOCHEMICAL CHARACTERIZATION OF THE DIFFERENTIATION MARKERS RECOGNIZED BY MABS

Introduction

There are two kinds of changes that contribute to the expression of differentiation markers specific for a particular cell type or lineage on normal hematopoietic cells or to the expression of transformation-associated antigens on retrovirus-transformed hematopoietic cells. One is the appearance of new surface markers due to enhanced synthesis at the transcriptional and/or translational level. The other is the alteration in the structure of carbohydrate groups attached to proteins or lipids at the posttranslational level. These changes probably are involved in the alteration of the interaction of particular hematopoietic cells with other types of hematopoietic cells, with the extracellular matrix or with stromal cells and in the different response to growth and differentiation factors.

Our initial attempt was to determine the nature and molecular weights of these differentiation markers in order to elucidate what kind of changes have occurred to these markers recognized by MAbs. The experimental approach

toward the characterization of these differentiation markers was via enzymatic digestion and chemical deglycosylation of the cell surface. Trypsin was used to test if proteins were portions of the antigens and endoglycosidase F and sodium-M-periodate were used to determine whether they were glycosylated. In addition, neuraminidase digestion was performed to test for the presence of sialic acid. Western blotting and biosynthetic labeling/immunoprecipitation techniques were utilized to determine the molecular weights of these markers.

Materials and Methods

Enzymatic Digestion

Trypsin. Cells were treated with 0.125% trypsin (Sigma) in TBS buffer, pH 5.0, for 1 hour at 37^{0}C. Cells were then washed, incubated with MAb supernatants followed by RIA.

Neuraminidase. Cells were treated with neuraminidase (from *Clostridium perfringens*; Sigma) at a concentration of 20 x 10^{6} cells/U in TBS buffer, pH 5.0, for 1 hour at 37^{0}C.

Endoglycosidase F. Cells were treated with Endo F at a concentration of 20 x 10^{6} cells/U in TBS buffer, pH 5.0, for 1 hour at 37^{0}C.

Chemical Deglycosylation

2.5 mM sodium-M-periodate in PBS was added to BM2

cells, 1.25 mM to 6C2 cells and 1.25 mM to MSB1 cells in TBS buffer, pH 5.0, for 1 hour at 37^0C.

Cell Membrane Solubilization

Unlabeled or ^{35}S-Methionine-labeled 50 x 10^6 cells were lysed in 1 ml lysis buffer containing 1.0% Triton X-100, 0.15 M NaCl, 0.01 M Tris-HCl, pH 8.0, 0.02% NaN_3, 2 mM PMSF and 100 KIU/ml aprotinin for 25 minutes on ice followed by the centrifugation at 12,000 x g for 15 minutes to get rid of the nuclear pellet.

Biosynthetic Labeling

50 x 10^6 cells were incubated with Methionine-free DMEM and 500 μ Ci L-^{35}S-Methionine for 7 hours at 37^0C. After two washes with cold TSA buffer (0.15 M NaCl, 0.01 Tris-HCl, pH 8.0, 0.02% NaN_3) the cells were then resuspended in 1 ml of lysis buffer.

Immunoprecipitation

Cell lysates were precleared with 50 μl protein G-sepharose 4B or protein A-sepharose 4B (Zymed) and 50 μl unrelated MAb supernatant overnight at 4^0C. The supernatant fluid of the cell lysates were recovered after centrifugation. 100 μl of precleared lysate was incubated with 100 μl MAb supernatant for 4 hours at 4^0C followed by the addition of 40 μl protein G-sepharose or protein

A-sepharose for 2 hours at 4^0C. The lysates were washed twice with washing buffer containing 0.1% Triton X-100/TSA, once with TSA buffer and then 0.05 M Tris-HCl, pH 6.8. After the last wash, the pellets were resuspended with 50 μl 2x SDS/sample buffer (2% SDS and 5% 2-mercaptoethanol) and boiled at 100^0C for 5 minutes.

SDS-PAGE

The supernatant fluids which were recovered from the centrifugation of the boiled immunoprecipitates in sample buffer were loaded at 20 K cpm per lane of a 10% acrylamide denaturing (SDS) discontinuous mini-gel. The SDS-PAGE was performed according to the method described by Laemmli (1970). Briefly, the gel was run at 25 mA of constant current per 0.75 mm slab gel on a Mini-PROTEIN II cell (BIO-RAD) for 45 minutes to 1 hour. Rainbow™ protein molecular weight markers (Amersham) were used for unlabeled cell lysates, whereas ^{14}C-methylated protein molecular weight markers (Amersham) were used for radiolabeled cell lysates.

Fluorography

After the electrophoresis was complete, the gel was fixed in 50% methanol and 10% acetic acid for 1 hour and then incubated with EN^{3}HANCE (Dupont) for 1 hour followed by two changes of tap water. The gel was dried in a slab-gel dryer (Hoefer Scientific Instruments) at 60^0C for 1 hour

onto a filter paper and exposed on a Kodak XAR-5 film for 1-3 days.

Silver Staining

The unlabeled proteins were detected by silver staining. Briefly, the polyacrylamide gel was fixed in fixing solution (50% methanol and 10% acetic acid) for 30 minutes and in destaining solution (5% methanol and 7% acetic acid) for 1 hour followed by 10% glutaraldehyde for 30 minutes. After 4 washes with water for 30 minutes each wash, the gel was stained with silver nitrate solution for 15 minutes and washed 5 times with water. Developer (25 ml of 0.5% w/v sodium citrate and 0.5% v/v 37% formaldehyde with 500 ml water) was then added and the gel was shaken vigorously until the bands appeared as desired. The gel was transferred to Kodak Rapid Fix (solution A) for 5 minutes and washed with water extensively. Eventually, the gel was dried in a gel dryer at 80^0C for 1 hour (Oakly *et al.*, 1980).

Results

Enzymatic Digestion and Chemical Deglycosylation

Three different cell lines, BM2/C3A, 6C2 and MSB1 were treated with trypsin, neuraminidase, Endo F and periodate oxidation respectively and then incubated with MAbs followed

by RIA to reveal whether or not the markers recognized by MAbs remained intact (Figure 4-1, 4-2 and 4-3).

All of the reactions of MAbs were knocked out by trypsinization, which indicates that proteins probably are the structural backbones of these markers. Deglycosylation with either periodate oxidation or Endo F digestion could decrease the binding specificities of MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10 to different degrees suggesting that these MAbs may be recognizing carbohydrate groups. In addition, treatment with neuraminidase showed that sialic acid also may contribute to the conformation of the antigenic determinants. Altogether, these data imply that the differentiation markers recognized by MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10 may be glycoproteins. Conversely, binding of 3F6-1E7 and 1F7-1A3 to the cells was not affected by deglycosylation and neuraminidase digestion, which suggests that the antigenic binding sites for 1F7-1A3 and 3F6-1E7 are localized on the polypeptide chain without the involvement of carbohydrate or sialic acid.

Interestingly, the reaction of MAb 1F7-1A3 with 6C2 cells was enhanced by neuraminidase digestion (Figure 4-2). This is possibly due to the masking of the antigenic determinant for MAb 1F7-1A3 by sialic acid in 6C2 cells.

The nature and antigenic determinants of the differentiation markers are summarized in Table 4-1.

Figure 4-1. Reactivities of MAbs with BM2 cells following enzymatic digestion and chemical deglycosylation. BM2 cells were treated with neuraminidase ($20x10^6$ cells/U), 0.125% trypsin, Endo F ($20x10^6$ cells/U) and 2.5mM sodium-M-periodate respectively for 1 hr at 37^oC. The reactivities of MAbs to the cells are expressed as the MAb binding indexes as determined by RIA.

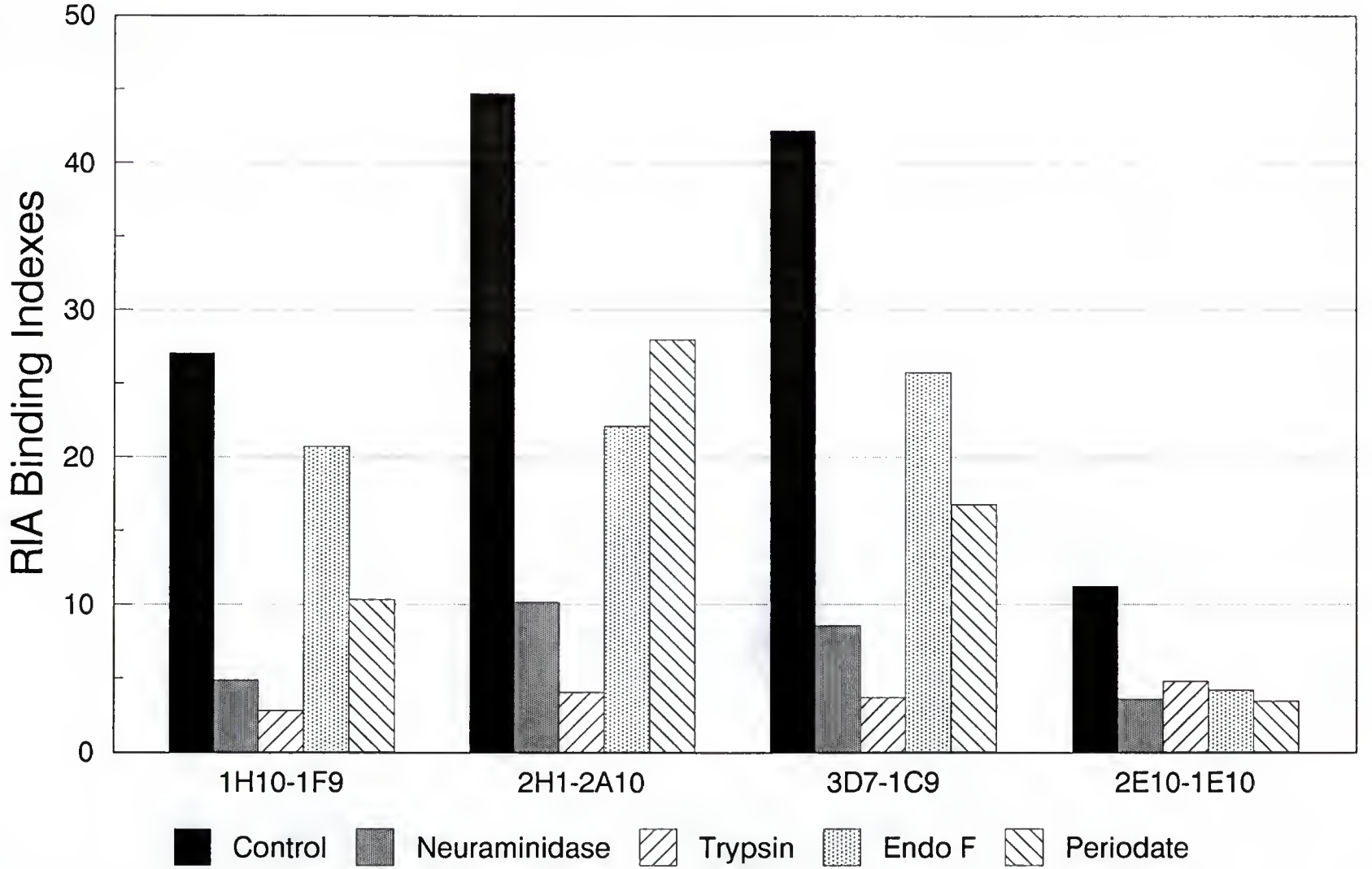

RIA Binding Indexes
50
40
30
20
10
0
1H10-1F9
2H1-2A10
3D7-1C9
2E10-1E10
Control
Neuraminidase
Trypsin
Endo F
Periodate

Figure 4-2. Reactivities of MAbs with 6C2 cells following enzymatic digestion and chemical deglycosylation. 6C2 cells were treated with neuraminidase ($20x10^6$ cells/U), 0.125% trypsin, Endo F ($20x10^6$ cells/U) and 1.25mM sodium-M-periodate respectively for 1 hr at 37^oC. The reactivities of MAbs to the cells are expressed as the MAb binding indexes as determined by RIA.

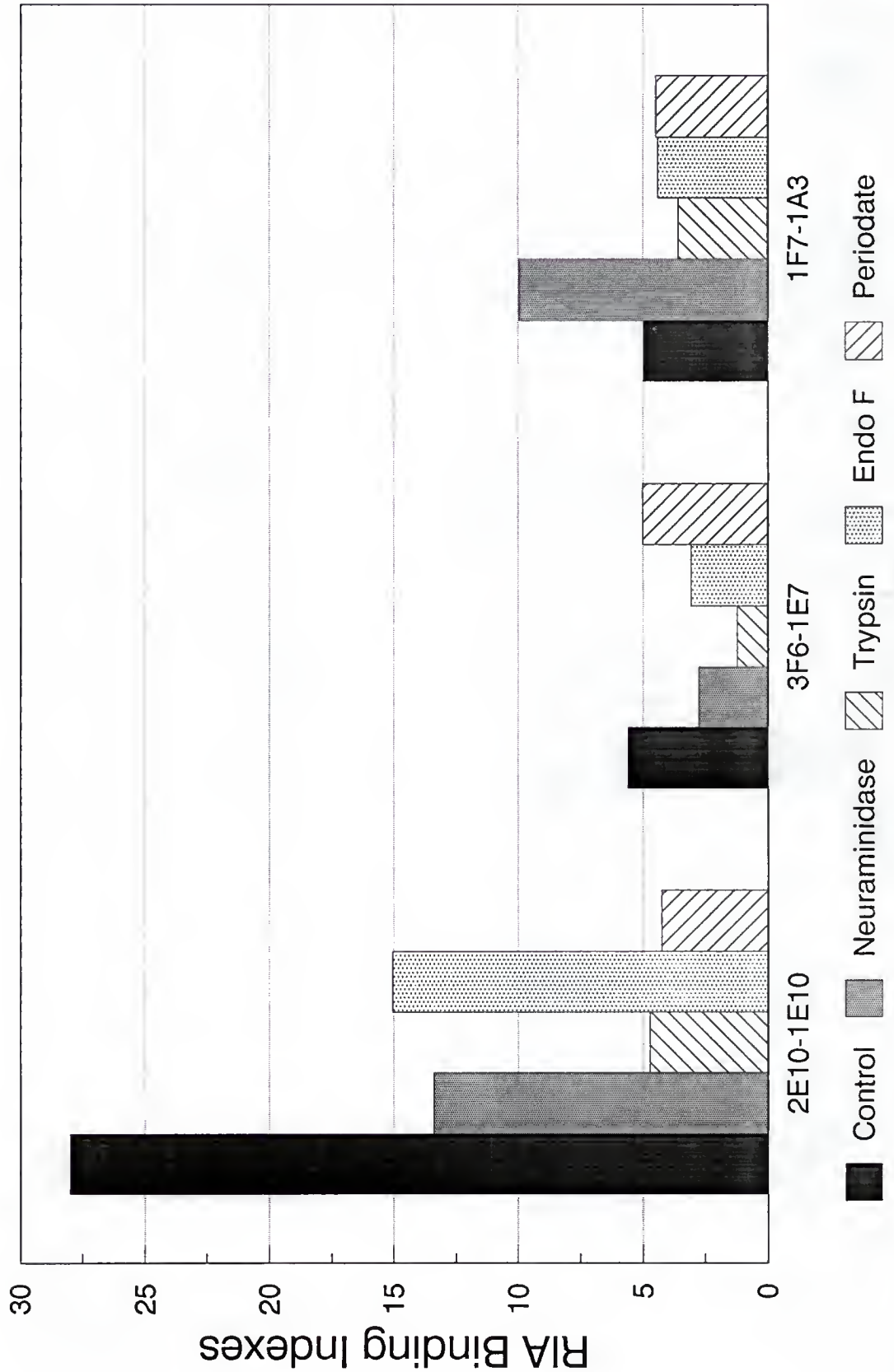

RIA Binding Indexes
30
25
20
15
10
5
0
2E10-1E10
3F6-1E7
1F7-1A3
Control
Neuraminidase
Trypsin
Endo F
Periodate

Figure 4-3. Reactivities of MAbs with MSB1 cells following enzymatic digestion and chemical deglycosylation. MSB1 cells were treated with neuraminidase ($20x10^6$ cells/U), 0.125% trypsin, Endo F ($20x10^6$ cells/U) and 1.25mM sodium-M-periodate respectively for 1 hr at 37^oC. The reactivities of MAbs to the cells are expressed by the MAb binding indexes as determined by RIA.

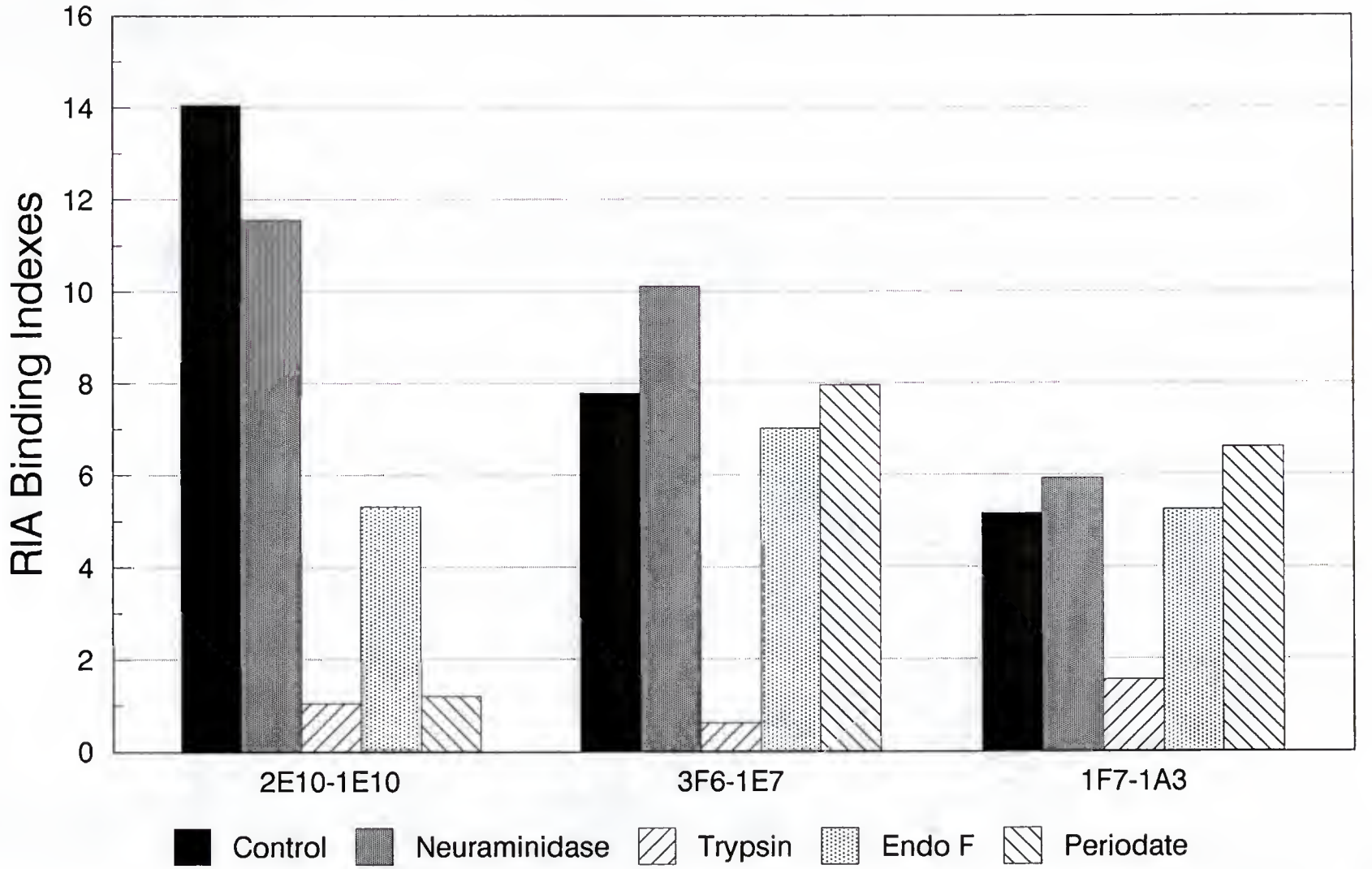

RIA Binding Indexes
16
14
12
10
8
6
4
2
0
2E10-1E10
3F6-1E7
1F7-1A3
Control
Neuraminidase
Trypsin
Endo F
Periodate

Table 4-1. The biochemical nature and antigenic determinants of the differentiation markers recognized by MAbs

MAb	Differentiation markers	
	Biochemical nature	Antigenic determinant
1H10-1F9	Glycoprotein	sialic acid +/-[a] CHO[b]
2H1-2A10	Glycoprotein	sialic acid +/- CHO
3D7-1C9	Glycoprotein	sialic acid +/- CHO
2E10-1E10	Glycoprotein	sialic acid +/- CHO
1F7-1A3	Glycoprotein ?	Protein
3F6-1E7	Glycoprotein ?	Protein

[a]+/-, with or without
[b]CHO, carbohydrate

Characterization of the Molecular Weights of These Markers

The attempts to characterize the molecular weights of these markers have not been very successful. The results of dot blots showed that the binding specificities of the MAbs to the cell lysates was decreased quite a bit after SDS denaturation (data not shown), which may explain why western blotting did not work.

Although the MAbs could be precipitated by protein G-sepharose (Figure 4-4), no specific bands of antigens were detected from the immunoprecipitates of ^{35}S-Methionine-labeled cell lysates (Figure 4-5, 4-6 and 4-7).

Discussion

Trypsinization, neuraminidase digestion and deglycosylation treatment could reduse the binding specificities of MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10. This suggests that the markers recognized by these MAbs are glycoproteins and that sialic acids with or without carbohydrates are contributing to the conformation of the antigenic determinants. Conversely, the antigenic determinants for MAbs 1F7-1A3 and 3F6-1E7 must be strictly localized on the polypeptide chain, since only trypsinization was able to inhibit their binding specificities. In addition, the antigenic determinant for MAb 1F7-1A3 is masked by sialic acid in 6C2 cells and

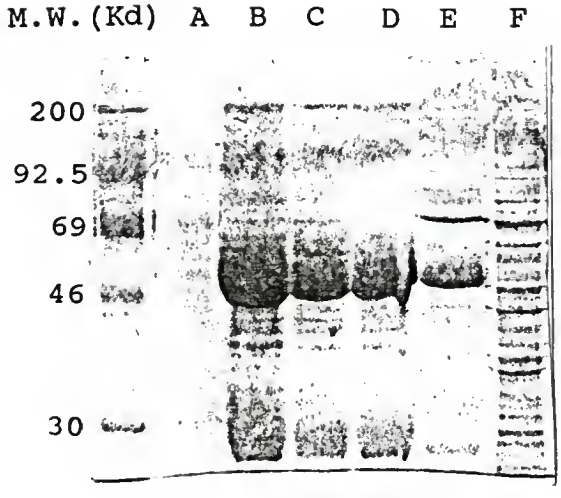


Figure 4-4. Silver staining of 10% SDS-PAGE analysis of immunoprecipitates obtained from unlabeled BM2/C3A cells. (A) protein G-sepharose alone, (B) 1H10-1F9 MAb sup + protein G-sepharose, (C) 2H1-2A10 MAb sup + protein G-sepharose, (D) 3D7-1C9 MAb sup + protein G-sepharose, (E) anti-BM2/C3A serum + protein G-sepharose, (F) BM2/C3A cell lysate only. The results showed that antibodies can be precipitated by ptrotein G-sepharose.

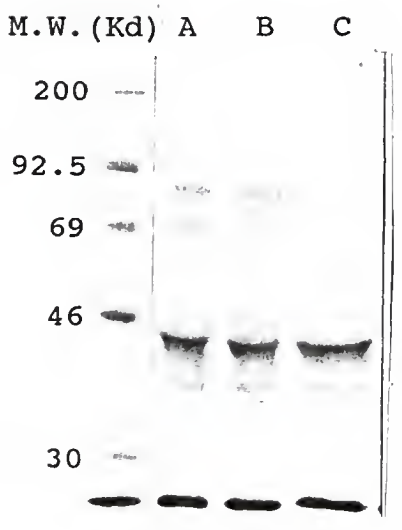


Figure 4-5. Fluorography of 10% SDS-PAGE analysis of immunoprecipitates obtained from ^{35}S-Methionine-labeled BM2/C3A cells. (A) 1H10-1F9 MAb sup + protein G-sepharose, (B) 2H1-2A10 MAb sup + protein G-sepharose, (C) 3D7-1C9 MAb sup + protein G-sepharose. No specific bands of cell lysates were precipitated by either MAb.

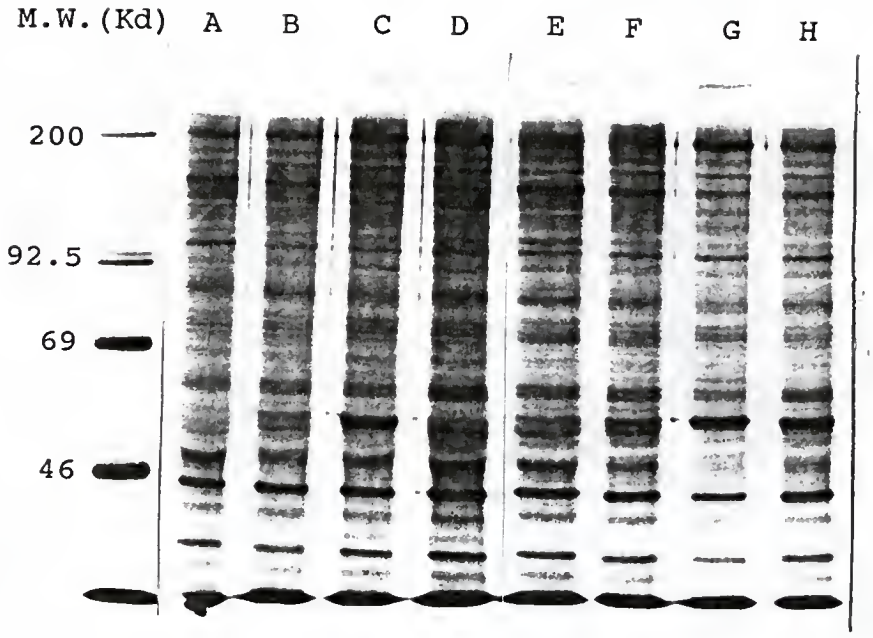


Figure 4-6. Fluorography of 10% SDS-PAGE analysis of immunoprecipitates obtained from ^{35}S-Methionine-labeled 6C2 cells. (A) 3F6-1E7 MAb sup + protein A-sepharose, (B) 2E10-1E10 MAb sup + protein A-sepharose, (C) 1F7-1A3 MAb sup + protein A-sepharose, (D) 1A8-1A2 MAb sup + protein A-sepharose, (E) 3F6-1E7 MAb sup + protein G-sepharose, (F) 2E10-1E10 MAb sup + protein G-sepharose, (G) 1F7-1A3 MAb sup + protein G-sepharose, (H) 1A8-1A2 MAb sup + protein G-sepharose. No specific bands of cell lysates were precipitated by either MAb.

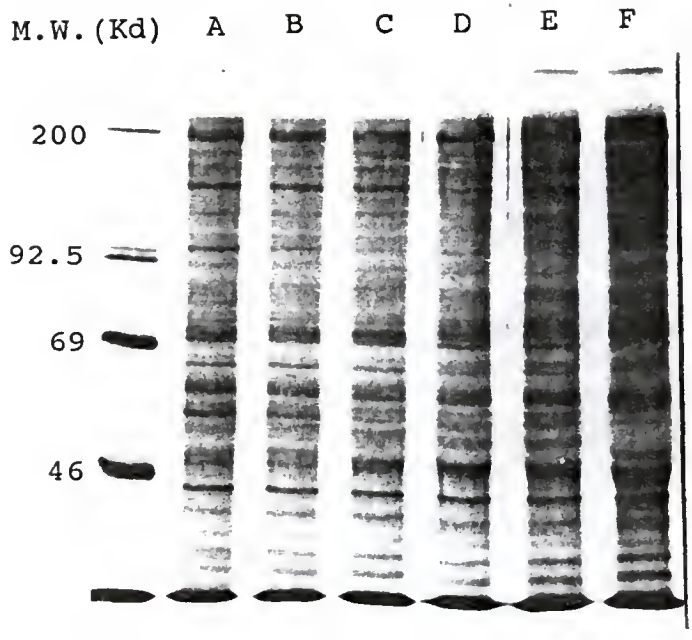


Figure 4-7. Fluorography of 10% SDS-PAGE analysis of immunoprecipitates obtained from ^{35}S-Methionine-labeled MSB1 cells. (A) 1F7-1A3 MAb sup + protein A-sepharose, (B) 1F7-1A3 MAb sup + protein G-sepharose, (C) 2E10-1E10 MAb sup + protein A-sepharose, (D) 2E10-1E10 MAb sup + protein G-sepharose, (E) 3F6-1E7 MAb sup + protein A-sepharose, (F) 3F6-1E7 MAb sup + protein G-sepharose. No specific bands of cell lysates were precipitated by either MAb.

possibly also in the normal BFU-E and CFU-E of the erythroid definitive lineage.

Cell surface sialic acid, comprising a group of *N*- and *O*-acyl as well as *O*-methyl and *O*-sulfate derivatives of neuraminic acid, is linked differently to galactose or *N*-acetylgalactosamine of glycoproteins and glycolipids (Schauser, 1988). Sialic acid has been shown to participate in several biological roles, such as the physicochemical properties of glycoproteins, the transport of glycoproteins inside the cell, the prevention of degradation of glycoconjugates, the survival of circulating cells and has been attributed a function in receptors for various substances infectious agents such as viruses, mycoplasmas and *pseudomonas*. In addition, several studies on transformed cells also suggest that cell surface sialic acid may play an important role in tumorigenicity and metastatic potential via its different properties including strong influences on platelet aggregation, tumor cell adhesion, the ability of tumor cells to implant in various organs, tumor cellular motility and deformability as well as invasiveness and immunogenicity of tumor cells (Yogeeswaran, 1983).

There are several ways in which sialic acid may contribute to the immunogenicity of normal cells and tumor cells. For instance, sialic acid may act as an antigen by itself; it may participate in the formation of antigenic

determinants along with other residues; or it can even mask antigen-binding sites on proteins, lipids and carbohydrates.

The deglycosylation experiments performed by Endo F digestion and periodate oxidation didn't result in the full inhibition of the reactions to MAbs. This may be due to: 1) Endo F doesn't work as well with the carbohydrate residues on cell surface glycoproteins as those on free glycoproteins. 2) The sublethal dosages of sodium-M-periodate, *i.e.*, 2.5mM for BM2/C3A cells, 1.25mM for 6C2 and MSB1 cells, are at a much lower concentration compared to the amount (10-50mM) was given to other cell types (Stein and Goldenberg, 1988; Mandeville *et al.*, 1987).

It was rather frustrating that these markers could not be precipitated by the MAbs at this point. This could be due to many factors, such as the alteration of the secondary and/or tertiary structure of the antigenic determinants by detergent solubilization, the decrease of binding affinity of MAbs by inappropriate pH in the lysis buffer and the interference of the electrophoresis pattern of specific immunoprecipitates by the nonspecifically bound cell lysates and the heavy chains of MAbs etc.

CHAPTER 5

CONCLUDING REMARKS

The major achievement in my project has been the generation of MAbs which are either specific for transformation-associated antigens or normal differentiation markers on avian hematopoietic cells. 1) MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 define transformation-associated antigens which are present preferentially on the AMV-transformed BM2 cell lines rather than on normal monocytic cells. The expression of these antigens is diminished after BM2 cells are induced to differentiate by PMA. 2) MAbs 1F7-1A3, 3F6-1E7 and 2E10-1E10 exhibit different specificities for normal differentiation markers. MAb 1F7-1A3 reacts with normal BFU-E and CFU-E, the AEV-transformed yolk sac cells and MSB1 cells. MAb 3F6-1E7 probably is specific for the stem cell and precursor cell populations during embryogenesis. In addition, the marker recognized by MAb 3F6-1E7 is also expressed on some transformed cells such as the AEV-transformed yolk sac cells, BM2 cell lines and MSB1 cells. MAb 2E10-1E10 defines a differentiation marker present on proliferating hematopoietic cells rather than terminally differentiated

cells, however, it starts appearing after the 4th day of embryogenesis and persists thereafter.

The specificities of these MAbs for avian hematopoietic cells is illustrated in Figure 5-1.

The biochemical characterization of the nature of these differentiation markers was conducted by enzymatic digestion and chemical deglycosylation. The binding of MAbs was knocked out by trypsinization, which indicates that proteins are the structural backbones of these markers. The removal of sialic acid by neuraminidase digestion as well as the deglycosylation with either periodate oxidation or Endo F could also reduce the binding specificities of MAbs 1H10-1F9, 2H1-2A10, 3D7-1C9 and 2E10-1E10 to different degrees. This suggests that the differentiation markers recognized by these MAbs are glycoproteins and that sialic acids with or without carbohydrates are contributing to the conformation of the antigenic determinants. In contrast, the binding specificities of MAbs 1F7-1A3 and 3F6-1E7 were not diminished either by deglycosylation or by neuraminidase treatment suggesting that a protein moiety contains the antigenic determinants for 1F7-1A3 and 3F6-1E7. However, it is yet to be determined whether or not these proteins are glycosylated.

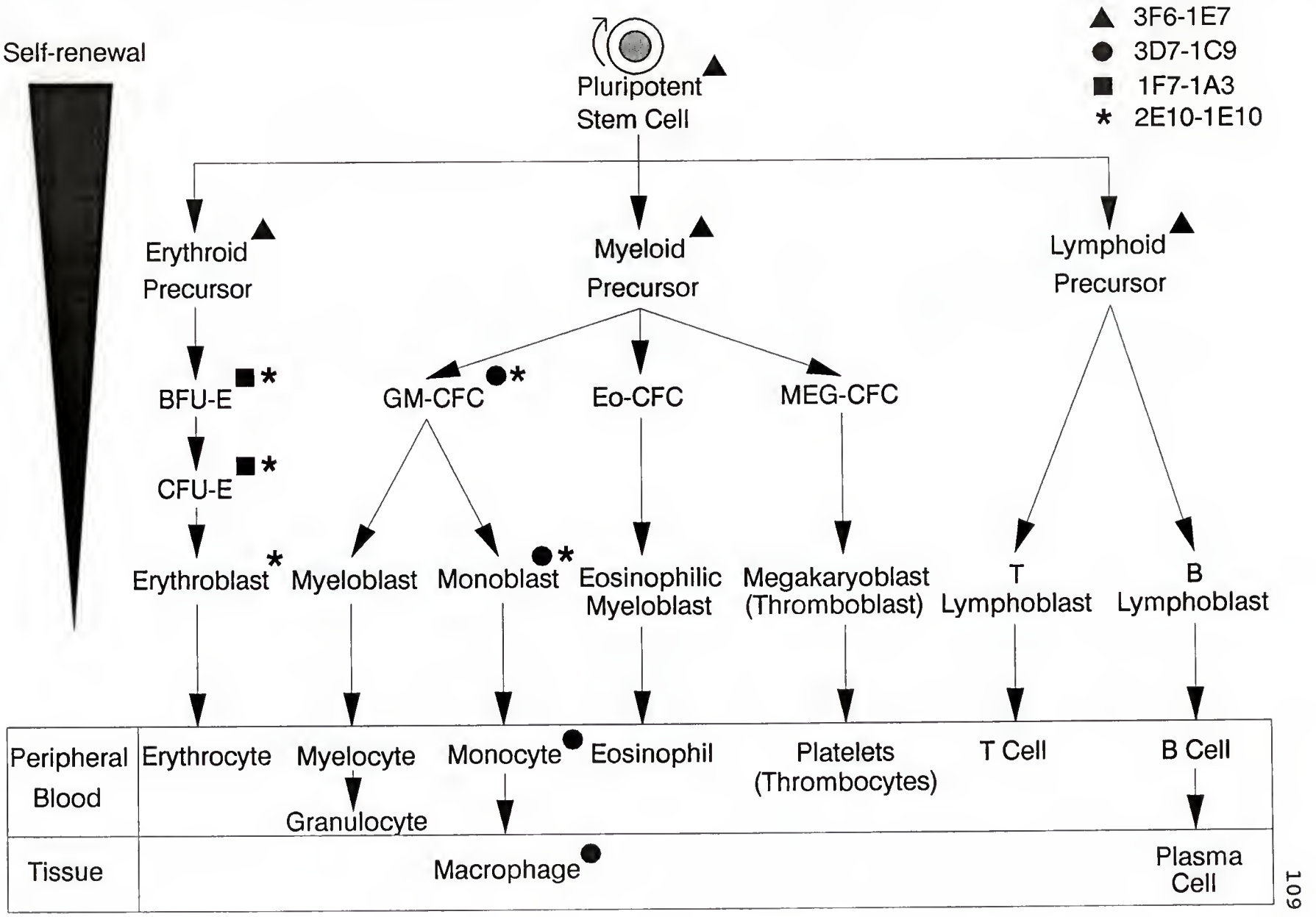
These MAbs will be useful for the diagnosis of the naturally occurring leukemia in the chicken. For instance, monocytic leukemia cells can be detected by monocytic cell-

specific MAbs such as 1H10-1F9, 2H1-2A10 and 3D7-1C9, and these MAbs provide us with the opportunity to test the therapeutic possibility of utilizing MAb-immunoconjugates for AMV-induced leukemia *in vivo*, whereas erythroleukemia cells will be recognized by 2E10-1E10 and not by other MAbs.

Some important questions remain to be elucidated by future investigators: 1) Is the expression of the normal differentiation markers recognized by MAbs 1F7-1A3, 2E10-1E10 and 3F6-1E7 essential for specific lineages and/or stages within the hematopoitic differentiation pathway? 2) I have demonstrated that the expression of transformation-associated antigens defined by MAbs 1H10-1F9, 2H1-2A10 and 3D7-1C9 is amplified in the AMV-transformed cells. Are these antigens the integral part of, or are merely associated with the *myb*-orchestrated transforming process? 3) How is the expression of these markers controlled? Is it due to the amplification at the transcriptional and/or translational level, or is it induced by posttranslational modifications of other differentiation markers such as aberrant glycosylation and/or sialylation?

To answer the above questions, it will be necessary to purify these differentiation markers followed by protein sequencing and structural characterization of their carbohydrate moieties by direct chemical methods such as NMR spectroscopy, mass spectrometry and methylation analysis. Once the protein sequences and the carbohydrate structures

Figure 5-1. Diagram of the specificities of MAbs for the hematopoietic cells. 3D7-1C9 is specific for the cells of monocytic lineage, 1F7-1A3 recognizes BFU-E and CFU-E, 2E10-1E10 detects a differentiation marker expressed on the proliferating hematopoietic cells after the 4th day of embryogenesis, whereas 3F6-1E7 probably reacts with the embryonic stem cell and precursor cell populations.


Self-renewal
Pluripotent Stem Cell
3F6-1E7
3D7-1C9
1F7-1A3
2E10-1E10
Erythroid Precursor
Myeloid Precursor
Lymphoid Precursor
BFU-E
CFU-E
GM-CFC
Eo-CFC
MEG-CFC
Erythroblast
Myeloblast
Monoblast
Eosinophilic Myeloblast
Megakaryoblast (Thromboblast)
T Lymphoblast
B Lymphoblast
Peripheral Blood
Erythrocyte
Myelocyte
Granulocyte
Monocyte
Eosinophil
Platelets (Thrombocytes)
T Cell
B Cell
Tissue
Macrophage
Plasma Cell

have been determined, their expression at the transcriptional, translational and posttranslational levels in normal versus tumor cells can be evaluated. The quest to understand tumor cells is definitely linked to our desire to know more precisely how the cell is able to direct a panel of cellular genes for normal proliferation and differentiation. The data presented in this dissertation represent for the first time a systematic attempt to compare how differentiation markers are expressed during hematopoietic development and how they may vary during the "chaos" provoked by oncoviruses.

" We cannot always assault the present problems of biology at will; we must remain alert to nature's clues and seize on them whenever and whereever they may appear--even if it be in a chicken."

--J.M. Bishop, Amer. Zool., 29 (1989), p665

REFERENCES

Abe, K., McKibbin, J.M. and Hakomori, S. (1983) J. Biol. Chem. 258, 11793-11797.

Akiyama, Y. and Kato, S. (1974) Bikens J., 17, 105-116.

Beug, H., Doederlein, G., Freudenstein, C. and Graf, T. (1982) J. Cell. Physiol. Suppl., 1, 195-207.

Beug, H. and Hayman, M.J. (1984) Cell, 36, 963-972.

Beug, H., von Kirchbach, A., Doederlein, G., Conscience, J.D. and Graf, T. (1979) Cell, 18, 375-390.

Bishop, J.M. (1983) Ann. Rev. Biochem., 52, 301-354.

Bister, K. and Jansen H.W. (1986) Adv. Cancer Res., 47, 99-187.

Bloch, A. (1984) Cancer Treat. Rep., 68,199-205.

Boettiger, D. and Durban, E.M. (1984) J. Virol., 49, 841-847.

Boucher, P., Koning, A. and Privalsky, M.L. (1988) J. Virol., 62, 534-544.

Boyle, W.J., Lampert, M.A., Li, A.C. and Baluda, M.A. (1985) Mol. Cell Biol., 5, 3017-3023.

Braylan, R.C., Benson, N.A., Nourse, V. and Kruth, H.S. (1982) Cytometry, 2, 337-343.

Bruns, G.A.P. and Ingram, W.M. (1973) Phil. Trans. Royal Soc. London, 266, 227-305.

Campbell, F. (1967) J. Morph., 103, 405-440.

Cordell, J.L., Falini, B., Ecber, W.N., Ghosh, A.K., Abdulaziz, Z., MacDonald, S., Pulford, K.A.F., Stein, H. and Mason, D.Y. (1984) J. Immunochem. Cytochem., 32, 219-229.

Cormier, F. and Dieterlen-Liévre, F. (1988) Develop., 102, 279-285.

Cruikshank, W.W., Berman, J.S., Theodore, A.C., Bernardo, J. and Center, D.M. (1987) J. Immunol., 138, 3817-3823.

Dieterlen-Liévre, F. (1988) In Vertebrate blood cells, Rowley, A.F. and Ratcliffe, N.A. (ed.), Cambridge University Press, London, pp. 257-336.

Dodge, W.H. and Hansell, C.C. (1978) Exp. Hemat., 6, 661-672.

Dodge W.H. and Moscovici, C. (1973) J. Cell. Physiol., 81, 371-386.

Dodge, W.H. and Sharma, S. (1985) J. Cell. Physiol., 123, 264-268.

Dodge, W.H., Silva, R.F. and Moscovici, C. (1975) J. Cell. Physiol., 85, 25-30.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Nature, 307, 521-527.

Durban, E.M. and Boettiger, D. (1981) Proc. Natl. Acad. Sci. USA, 78, 3600-3604.

Enrietto, P.J. and Wyke J. A. (1983) Adv. Cancer Res., 39, 269-314.

Evans, A.E., Gerson, J. and Schnanfer, L. (1976) Natl. Cancer Inst. Monogr., 44, 49-54.

Fenner, F. (1976) Intervirol., 7, 1-115.

Fresney, R.I. (1985) Anticancer Res., 5, 111-130.

Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M.J. and Vennström, B. (1983) Cell, 32, 227-238.

Fukuda, M. (1985) Biochim. Biophys. Acta, 780, 119-150.

Fung, Y.-K.T., Lewis, W.G., Crittenden, L.B. and Kung, H.-J. (1985) Cell, 33, 357-368.

Gandrillon, O., Jurdic, P., Pain, B., Desboies, C., Madjar, J.J., Moscovici, M.G., Moscovici, C. and Samarut, J. (1989) Cell, 58, 115-121.

Gazzolo, L., Moscovici, C., Moscovici, M.G. and Samarut, J. (1979) Cell, 16, 627-638.

Gazzolo, L., Samarut, J., Bouabdelli, M. and Blanchet, J.P. (1980) Cell, 22, 683-691.

Gilmore, T., DeClue, J.E. and Martin, G.S. (1985) Cell, 40, 609-618.

Gootwine, E., Webb, C.G. and Sachs, L. (1982) Nature, 299, 63-65.

Graf, T. and Beug, H. (1978) Biochem. Biophys. Acta, 516, 269-299.

Graf, T. and Stéhelin, D. (1982) Biochim. Biophys. Acta, 651, 245-271.

Graf, T., von Kirchbach, A. and Beug, H. (1981) Exp. Cell Res., 131, 331-343.

Gregory, C.J. and Eaves, A.C. (1978) Blood, 51, 527-537.

Hanafusa, H. (1977) In Comprehensive virology, 10, Franenkel-Conrat, H. and Wagner R. (ed.), Plenum Press, New York, pp. 401-483.

Hasek, M. and Hraba, T. (1955) Nature, 175, 764-765.

Hayman, M.J. and Beug, H. (1984) Nature, 309, 460-462.

Hayman, M.J., Beug, H. and Savin, K.W. (1982) J. Cell. Biochem., 18, 351-362.

Hayward, W.S., Neel, B.G. and Astrin, S.M. (1981) Nature, 290, 475-479.

Ingram, V.M. (1972) Nature, 235, 338-339.

Jurdic, P., Bouabdelli, M., Moscovici, M.G. and Moscovici, C. (1985) Virology, 144, 73-79,

Jurdic, P., Moscovici, C., Pessano, S., Bottero, L. and Rovera, G. (1982) J. Cell. Physiol. Suppl., 2, 85-95.

Kahn, P., Frykberg, L., Brady, C., Stanley, Il, Beug, H., Vennström, B. and Graf, T. (1986) Cell, 45, 349-356.

Klempnauer, K.-H., Symonds, G., Evan, G.I. and Bishop, J.M. (1984) Cell, 37, 537-547.

Kohler, G. and Milstein, C. (1975) Nature, 256, 495-497.

Kornfeld, S. Beug, H., Doederlein, G. and Graf, T. (1983) Exp. Cell Res., 143, 383-394.

Laemmli, U.K. (1970) Nature, 227, 680-685.

Leutz, A., Beug, H. and Graf, T. (1984) EMBO J., 3, 3191-3197.

Lim, R., Hicklin, D.J., Ryken, T.C., Han, X-M., Liu, K-N., Miller, J.F. and Baggenstoss, B.H. (1986) Cancer Res. 46, 5241-5247.

Mandeville, R., Dumas, F., Amarouch, A., Sidrac-Ghali, S., Walker, M.C., Zelechowska, M., Adjukovic, I. and Grouix, B. (1987) Hybridoma, 6, 441-451.

Metcalf, D. and Moore, M.A.S. (1971) In Frontiers of biology, 24, Neuberger, A. and Tatum, E.L. (ed.), North-Holland Publishing Company, Amsterdam, pp. 1-9.

Miller, M.M., Goto, R. and Clark, D. (1982) Dev. Biol., 94, 400-414.

Moscovici, C. and Gazzolo, L. (1982) Adv. Viral Oncol., 1, 83-106.

Moscovici, C., Gazzolo, L. and Moscovici, M.G. (1975) Virology, 68, 173-181.

Moscovici, C. and Vogt, P.K. (1968) Virology, 35, 487-497.

Moscovici, C., Zeller, N. and Moscovici, M.G. (1982) In Expression of differentiated functions in cancer cells, Revoltella, R.F., Basilico, C., Gallo, R.C., Potori, G.M. Rovera, G. and Subak-Sharpe, J.H. (ed.), Raven Press, New York, pp. 435-449.

Moscovici, M.G., Jurdic, P., Samarut, J., Gazzolo, L. Mura, C.V. and Moscovici, C. (1983) Virology, 129, 65-78.

Moscovici, M.G. and Moscovici, C. (1980) In In vivo and in vitro erythropoiesis: The friend system, Rossi, G.B. (ed.), Elsevierl North-Holland Biochemical Press, Amsterdam, pp. 503-514.

Moscovici, M.G. and Moscovici, C. (1983) Proc. Natl. Acad. Sci. USA, 80, 1421-1425.

Nelson, C.H., Allison, J.P., Kline, K. and Sanders, B.G. (1982) Cancer Res., 42, 4625-4630.

Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980) Anal. Biochem., 105, 361-363.

Old, L.J. (1981) Cancer Res., 41, 361-375.

Payne, G.S., Bishop, J.M. and Varmus, H.S. (1982) Nature, 295, 209-213.

Payne, L.N. and Powell, P.C. (1984) In Physiology and biochemistry of the domestic fowl. Freeman, B.M. (ed.), Academic Press, Inc., London, pp. 277-321.

Pessano, S., Gazzolo, L. and Moscovici, C. (1979) Microbiologica, 2, 379-392.

Pierce, G.B., Aguilar, D., Hood, G. and Wells, R.S. (1984) Cancer Res., 44, 3987-3996.

Pierce, G.B., Lewis, S.H., Miller, G., Moritz, E. and Miller, P. (1979) Proc. Natl. Acad. Sci. USA, 76, 6649-665.

Podesta, A.H., Mullins, J., Pierce, G.B. and Wells, R.S. (1984) Proc. Natl. Acad. Sci. USA, 81, 7608-7611.

Ponder, B.A. and Wilkinson, M.M. (1981) J. Histochem. Cytochem., 29, 981-984.

Privalsky, M.L. and Bishop, J.M. (1984) Virology, 135, 356-368.

Privalsky, M.L., Sealy, L, Bishop, J.M., McGrath, J.P. and Levinson, A.D. (1983) Cell, 32, 1257-1267.

Sachs, L. (1986) Sci. Am. 254, 30-37.

Samarut, J., Blanchet, J.P., Godet, J. and Nigon, V. (1976) Ann. Inst. Pasteur (Paris), 127C, 873-888.

Samarut, J., Blanche, J.P. and Nigon, V. (1979) Dev. Biol., 72, 155-166.

Samarut, J. and Bouabdelli, M. (1980) J. Cell. Physiol., 105, 553-565.

Samarut, J. and Gazzolo, L. (1982) Cell, 28, 921-929.

Sanders, B.G., Allison, J.P., and Kline, K. (1982) Cancer Res., 42, 4532-4539.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H. and Vennström, B. (1986) Nature, 324, 635-640.

Schauer, R. (1988) In The molecular immunology of complex carbohydrates. Wu, A.M. (ed.), Plenum Press, New York, pp. 47-72.

Schmidt, J.A., Marshall, J., Hayman, M.J., Doderlein, G. and Beug, H. (1986) Leuk. Res., 10, 257-272.

Sealy, L., Privalsky, M.L., Moscovici, M.G., Moscovici, C. and Bishop, J.M., (1983) Virology, 130, 155-178.

Shih, C.-K., Linial, M., Goodenow, M.M. and Hayward, W.S. (1984) Proc. Natl. Acad. USA, 81, 4697-4701.

Shinitzky, M. (1984) Biochem. Biophys. Acta, 738, 251-261.

Silva, R.F., Dodge, W.H. and Moscovici, C. (1976) J. Cell. Physiol., 83, 187-192.

Sorrell, J.M. and Weiss, L. (1980) Anat. Res., 197, 1-19.

Sporn, M.B., Roberts, A.B., Wakefield, L.M. and Assoian, R.K. (1986) Science, 233, 532-534.

Stanbridge, E.J. Der, C.J., Doersen, C-J., Mishimi, R.Y., Puhl, D.M., Weissman, B.E. and Wilkinson, J.E. (1982) Science, 215, 252-259.

Stein, R. and Goldenberg D.M. (1988) Hybridoma, 7, 555-567.

Symonds, G., Klempnauer, K.-H., Evan, G.I. and Bishop, J.M. (1984) Mol. Cell. Biol., 4, 2587-2593.

Szenberg, A. (1977) Adv. Exp. Med. Biol., 88, 3-11.

Till, J.E. and McCulloch, E.A. (1961) Radiat. Res., 14, 213-222.

Trembicki, K.A. and Dietert, R.R. (1985) J. Exp. Zool., 235, 127-134.

Vogt, P.K. (1969) In Fundamental techniques in vorology, Habel, K. and Salzman, N.P. (ed.), Academic Press, New York, pp. 198-211.

Wallach, D.F.H. (1968) Proc. Natl. Acad. Sci. USA, 61, 868-874.

Weston, K. and Bishop, J.M. (1989) Cell, 58, 85-93.

Yamamoto, T., Hihara, H., Nishida, T., Kawai, S. and Toyoshima, K. (1983) Cell, 34, 225-232.

Yogeeswaren, G. (1983) Adv. Cancer Res., 38, 289-350.

Zenke, M., Kahn, P. Disela, C., Vennström, B., Leutz, A., Keegan, K., Hayman, M.J., Choi, H.-R., Yew, N. Engel, J.D. and Beug, H. (1988) Cell, 52, 107-119.

BIOGRAPHICAL SKETCH

Juinn-Lin G. Liu was born in Kao-Shong, Taiwan, on October 23, 1960. He grew up with cartoons, baseball and traffic. He went to the Department of Veterinary Medicine, National Taiwan University, in September, 1978. During his fifth year as an intern, he discovered two interesting cases which were never been found in Taiwan before. One was a female Akita dog which suffered from congenital intestinal lymphangiectasia; the other case was a male Pit bulldog with the TVT (transmissible venereal tumor) developed on the skin instead of in the genital regions. It was possibly due to been bitten by another fight dog which had the involvement of the TVT in the mouth cavity. Shortly after he received his D.V.M. degree in June, 1983, there was another female Pit bulldog had exactly the same skin-type TVT. Before he had any opportunity to do further investigation, he was drafted by the Chinese Marine Corps to fulfill his two-year military service and he was overwhelmed with military and physical training as well as brain wash. Somehow he managed to remain intact with the exception that he became allergic to beer and then came to the University of Florida to pursue his Ph.D. degree in the Department of Pathology and Laboratory Medicine, in August, 1985 until

now. He has accepted a postdoctoral position in the Department of Molecular Biology and Microbiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio. He is scheduled to "launch" in January, 1990 despite the forecast of one foot of snow waiting for him. Yes! Your guess is as good as mine, he has never seen snow before.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Carlo Moscovici, Chair
Professor of Pathology and
Laboratory Medicine

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Klein
Professor of Pathology and
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward K. Wakeland
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Associate Professor of Pathology
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Gradute School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1990

Dean, College of Medicine

Dean, Gradute School